UNIVERSITY OF NOTTINGHAM

Characterization and comparison of *Campylobacter* Bacteriophages

A PhD Thesis by

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ABSTRACT

Members of the genus *Campylobacter* are a major cause of food-borne disease worldwide. They can colonize the intestinal mucosa of poultry, to high levels leading to contamination of meat, at slaughter. Their numbers can be reduced in different ways including chicken treatment with bacteriophages. For such treatments to be successful, in depth understanding of the bacteriophage that infects and kills campylobacters is vital. The work in this thesis describes: isolation and comprehensive characterisation of bacteriophage candidates for future therapy applications.

In order to increase the available stocks of characterized candidate bacteriophage, a number of attempts were made to isolate bacteriophages from poultry excreta. The new isolates together with some uncharacterized phages from our laboratory stocks were characterized with respect to their host range and genomic size. Some bacteriophages preparations in previous studies showed genomes of different sizes and a number of attempts were done for their separation. This raised questions about the relationship between the two different sized genomes. Prior to this work, a co isolate pair had been successfully separated and the sequence of the larger genome, CP220, was determined. Part of the work here, was performed to extend this study by obtaining the sequence of the smaller co isolate, CPX and compare it to CP220. They did not appear to have any identifiable relationship at the genetic level, but the availability of the CPX sequence will further extend our knowledge of bacteriophage genetics and this phage has clear therapeutic potential. Attempts were also made to separate and characterize a second co-isolate pair but these were unsuccessful.

The availability of the DNA sequence of CP220 allowed a much closer molecular characterisation and comparison of *Campylobacter* phage genomes, than had previously been possible. One area that was investigated in this study was the presence of repeat regions identified in the CP220 genome, which were amplified by PCR, but could not be cloned in *E. coli.* Furthermore, genes encoding potential lysins were identified in the CP220 genome and they were amplified, cloned and attempts were made to express the proteins, which may have potential therapeutic value. One gene product was successfully expressed and showed evidence of lytic activity on *Campylobacter* and other bacterial genera.

In summary, this thesis describes a much closer examination of molecular biology of *Campylobacter* bacteriophage than had previously been possible, including the determination of the sequence CPX phage.

Dedication

I dedicate this thesis to;

My parents for encouraging me to continue my higher education,

My brothers and sisters without whose support, it would not have been possible to continue this work,

My beloved children Abdullah and Haneen for inspiring me.

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CHAPTER ONE

INTRODUCTION

1.1 The Genus *Campylobacter*

1.1.1 General Characteristics

Campylobacters are Gram-negative non-saccharolytic, non-spore forming bacteria (Newell and Fearnley, 2003). The cells have a spiral shape with approximate dimensions 0.2-0.8 μm wide and 0.5-5.0 μm long. Most *Campylobacter* spp. are motile with a single polar unsheathed flagellum at one or both ends of the cell. Some, like *C. gracilis,* are non-motile and others, like *C. showae*, have multiple flagella (Debruyne *et al.,* 2008). Campylobacters are found as part of the normal gastrointestinal flora of many domestic animals and pets (Corry and Atabay 2001; Shen *et al.*, 2001). Campylobacters were not differentiated from "true" *Vibrio* spp. until 1963 by Sebald and Vèron (1963). This differentiation was due to many reasons including: low G+C DNA base composition, their non-fermentative metabolism and their microaerophilic growth requirement (On, 2001). *Campylobacter* species are fastidious in their *in vitro* growth requirements and have little or no global stress response mechanisms against harsh environmental conditions (Murphy *et al.*, 2006). The G+C content of the DNA ranges from 29 to 47 mol %. The number of distinct *Campylobacter* species and subspecies varies from author to author, due to new species being discovered and some being reclassified in to different genera. Currently there are 24 species with eight subspecies assigned to the genus *Campylobacter* (Debruyne *et al.*, 2008; 2009, 2010a and b; Rossi *et al.,* 2009; Zanoni *et al.*, 2009; Vandamme *et al.,* 2010). The *Campylobacter* species currently recognised are listed in Table 1.1. *Campylobacter jejuni* is recognised as the most prevalent human enteropathogenic *Campylobacter* species, followed by *C. coli*. Most of this review is concentrated on these two species

because they account for the majority of human disease. *Campylobacter jejuni* has two subspecies; *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* that differ in their biochemical characteristics (Debruyne *et al.*, 2008). The complete genome sequence of *C. jejuni* NCTC 11168 was first published by Parkhill *et al.* (2000).

Table 1.1 *Campylobacter* species

Campylobacter species	Sources	References
C. fetus	Sheep, cattle, ungulates, fowl, reptiles and humans	McFadyean and Stockman 1913
C. hyointestinalis	Pigs, hamsters, cattle faeces, deer and human blood	Debruyne *et al.* 2008
C. lanienae	Faecal samples of abattoir workers, pigs	Logan *et al.* 2000
C. sputorum	Cattle faeces, sheep, humans, bulls, pigs	On *et al.* 1998 Atabay *et al.* 1997
C. mucosalis	Pigs, porcine oral cavity	Tyrrell *et al.* 2003
C. concisus	Humans	Tanner *et al.* 1981
C. curvus		
C. rectus	Humans	Tanner *et al.* 1981
C. gracilis		
C.showae	Humans	Etoh *et al.* 1993
C. hominis	Humans	Lawson *et al.* 1998
C. jejuni	Cattle faeces, humans poultry	Smith and Orcutt 1927 Atterbury *et al.*, 2003a
C. coli	Cattle faeces, humans	Smith and Orcutt 1927
	Pigs	Jensen *et al.*, 2006
C. lari	Seagulls, river water, shellfish, humans	Endtz *et al.* 1997
C. insulaenigrae	Seals, porpoise, elephant seals	Stoddard *et al.* 2007
C. canadensis	Whooping cranes	Inglis *et al.* 2007
C. upsaliensis	Humans, cat faeces, dog faeces	Debruyne *et al.* 2008
C. helveticus		
C. ureolyticus	Intestinal biopsy specimens and faeces samples of children with newly diagnosed Crohn's disease	Vandamme *et al.* 2010
C. cuniculorum	Rabbit	Zanoni *et al.*, 2009
C. peloridis	Shellfish and humans	Debruyne *et al.*, 2009
C. avium	Poultry	Rossi *et al.*, 2009
C. subantacticus	Wild birds in the sub-Antarctic region	Debruyne *et al.*, 2010b
C. volucris	Gulls	Debruyne *et al.*, 2010a

1.1.2 Biochemical Properties

Many of the *Campylobacter* species (Table 1.1) have similar biochemical properties, such as the ability to reduce fumarate to succinate and the absence of hippuricase, with the exception of *C. jejuni* in the case of the latter characteristic (Debruyne *et al.*, 2008). All *Campylobacter* species are oxidase positive, (except *C. gracilis,* which obtains energy from amino acids or tricarboxylic acid cycle intermediates), as they are unable to ferment or oxidize carbohydrates (Vandamme, 2000). Moreover, most of the species cannot reduce nitrate and they give negative results in the methyl red reaction and indole tests (Debruyne *et al.*, 2008).

1.1.3 Isolation Techniques

Campylobacters are fastidious in their growth requirements and very sensitive to stresses, thus their isolation is challenging. The use of selective media for *Campylobacter* isolation is recommended, depending on the sample type (Hu and Kuo, 2011). Different workers isolated Campylobacters using different media containing different combinations of selective agents including: cefoperazone, cycloheximide, trimethoprim, rifampicin, vancomycin or polymyxin B. A number of media that are based on blood and antibiotics such as: Skirrow's. Butzler's and CampyBAP are considered as the commonly used media for *Campylobacter* isolation (Baylis *et al.*, 2000; Granato, *et al.*, 2010). Some selective agents have inhibitory effects on damaged cells, thus a suspension is first made in basal broth followed by the addition of a selective agent after a short period of incubation, with a gradual increase of incubation temperature between 37 and 41.5 °C (ISO; 1995, 2006).

To improve the recovery of these bacteria from samples having low numbers, enrichment by filtration can be beneficial (Hu and Kuo, 2011). In addition, enrichment is required in samples having injured cells resulting from different stresses during food processing and storage (Rosenquist *et al.*, 2006). The use of filtration was described by Butzler *et al.* (1973) for the isolation of *Vibrio* from stools of children and adults having diarrhoea. Filtrates of faecal suspensions were passed through membrane filters (mean pore size 0.65 μm) and then cultured on non-selective agar. This step is not required for isolating campylobacters from chicken excreta samples as the use of selective broth and direct plating on selective media resulted in maximum recovery values (Mason *et al.*, 1999).

Routine selective methods developed to isolate *C. jejuni* and *C. coli* from human patients in diagnostic laboratories, are not considered practical for the large number of samples that are processed daily, so their numbers may be underestimated. Also, they are generally unsuitable for the isolation of the other members of the *Campylobacter* genus that cause human disease. Methods that are suitable for the isolation of these include the use of detection methods that avoid selective isolation media such as the membrane method of Steele and McDermott (1984) or the method used by Lastovica *et al.* (1989) that can detect a greater variety of *Campylobacter* species as well as *Arcobacter* spp.. Cefoperazone amphotericin teicoplanin (CAT) agar was developed by Aspinall *et al.* (1993) through modifications of modified charcoal cefoperazone deoxycholate agar (mCCDA) with the use of different antibiotic levels for the isolation of *C. upsaliensis* as well as the thermophillic campylobacters. This

medium was used by Corry and Attabay (1997) in comparison to mCCDA, for the isolation of a number of *Campylobacter* and *Arcobacter* strains and its efficiency in this respect was proved. In addition, Aspinall *et al.* (1996) reported that the use of CAT media, rather than the membrane filtration techniques on Blood Agar (BA) plates, resulted in fewer competitors.

A large number of comparisons of the various enrichment and cultural methods have been described in the literature for the isolation and enumeration of campylobacters from different sources. A standard method was published by The International Organization for Standardization (ISO) which included the use of Bolton broth for enrichment step followed by culturing on mCCDA selective media and any alternative medium to be chosen by the person performing the experiment (ISO 10272-1, 2006). Lynch *et al.* (2010) developed a universal protocol for growing 17 species and three subspecies of *Campylobacter*. It involves an enrichment step for 24 h in *Campylobacter* enrichment broth (CEB), at 37 °C under a low oxygen atmosphere containing 2.5 % O_2, 7 % H_2, 10 % CO_2 and 80.5 % N_2, followed by centrifugation and filtration on to Anaerobe Basal Agar (ABA).

Different studies have been performed to compare direct plating and enrichment. One such study involved a comparison of mCCDA, Karmali and Preston agar with or without enrichment in Exeter broth for *Campylobacter* isolation from the caecal contents of chickens. It was found that enrichment did not improve the isolation rate. For direct plating, mCCDA showed a higher isolation rate than Karmali and Preston agars (Rodgers *et al.*, 2010). Similar findings were reported by Kiess *et al.* (2010) for isolating campylobacters from

broiler litter using a different set of media. Regardless which media is used, direct plating proved more effective for the recovery of campylobacters compared with the use of an enrichment step that was performed in CEB. All of the five media compared: Campy-Line agar (CLA), Campy-Cefex agar (CCA), modified CCA, Campylobacter agar plates (CAP) and mCCDA showed similar isolation rates. Most recently, Ugarte-Ruiz *et al.* (2012) described a comparison between direct plating on mCCDA or Campyfood agar (CFA) with enrichment using Bolton or Preston broths for the isolation of campylobacters from poultry faeces, neck skin and packed fresh meat. They concluded that direct plating is better than enrichment in samples like faeces and neck skin because of the presence of high contamination levels. For samples with less contamination such as poultry meat, then Preston broth showed better recovery rates than Bolton broth recommended by the ISO standard. They also, reported that plating on mCCDA was not significantly different from CFA, yet direct plating on CFA was better because of the ease of colony identification.

Another recent comparison was provided by Habib *et al.* (2011), who compared direct plating and enrichment using three different broths (Preston, Bolton and Campyfood) and three selective media including mCCDA, CFA and Brilliance Campy count agar (BCC) for isolating campylobacters from chicken meat samples. Bolton broth was a good choice of enrichment broth for *Campylobacter* growth and competitor inhibition. Yet, Preston broth performed better than Bolton broth regardless of the subsequent plating media. Isolation

using CFA and BCC media was easy and precise and they were good alternative media to mCCDA.

The optimum temperature for *C. jejuni* is generally agreed to be between 37 °C and 43 °C. It lacks the ability to grow below 30 °C and above 47 °C under a microaerobic atmosphere. These gaseous conditions can be generated by using a gas generating kit, Campy-pack system or an evacuation replacement method where the air is removed from the incubating jar and replaced with a gas mix containing 80% nitrogen, 15% carbon dioxide and 5% oxygen (Barros-Velázquez *et al.*, 1999).

1.1.4 *Campylobacter* Confirmatory Tests

Different methods can be used to distinguish campylobacters from other bacterial genera following their isolation on selective media. On mCCDA, campylobacters appear as flat, moistened greyish colonies.

Gram stain can be used to differentiate campylobacters which are Gram-negative from Gram-positive bacteria, based on the chemical and physical properties of the cell wall. In addition to that, their morphology and motility can be examined using phase contrast microscopy. Oxidase test is another biochemical technique that is used to identify the campylobacters that produce cytochrome oxidase from those that do not.

Latex tests, using particles coated with antibodies that agglutinate in the presence of *Campylobacter* antigens, can be performed to confirm the presence of C. *jejuni* or *C. coli* colonies picked from agar plates (Nachamkin and

Barbagallo, 1990). Commercially available systems include: Campyslide system, Meritec-campy system and Microscreen methods (Hazeleger *et al.*, 1992).

1.1.5 Discrimination Between *Campylobacter* Species

A number of phenotypic and genotypic methods have been proposed and used by different scientists in order to differentiate between bacteria at the species and subspecies level, each with its own advantages and disadvantages.

Biotyping methods are based on variation in the metabolic activities of the species to enable discrimination (Eberle and Kiess, 2012). A number of tests may be used to differentiate between different species of *Campylobacter*. For example, a positive hippurate test distinguishes *C. jejuni* from other campylobacters, because it alone produces the hippuricase enzyme. Catalase production, sensitivity to nalidixic acid or cephalothin, indoxyl acetate hydrolysis and growth at different temperatures are all tests which are used to differentiate between the other species (Vandamme, 2000). Different systems have been proposed to distinguish between *C. jejuni*, *C. coli* and *C. lari*. The system proposed by Skirrow and Benjamin (1980) was based on the ability of the species to grow at 25 °C and 43 °C, susceptibility to nalidixic acid, hippurate hydrolysis and hydrogen sulphide production in iron-containing media. On the other hand, another system developed by Lior (1984) was based on hippurate hydrolysis, rapid production of hydrogen sulphide and DNA hydrolysis.

Whilst many different molecular methods have been developed to improve bacterial identification to species level, the fairly recent availability of many genome sequences meant that PCR amplification has become the method of choice to distinguish between species, as it is quick and cheap. Thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) from clinical samples were discriminated by Eyers *et al.* (1993) using PCR amplification of 23S rDNA gene. Multiplex PCR is a rapid and easy method that has a high sensitivity and specificity. It is based on using specific primers designed from known DNA sequences to produce PCR fragments of a size present only in members of the species to be distinguished. Several sets of primers are used, hence the term "multiplex". This method was used by Wang *et al.* (2002) to distinguish between different species of *Campylobacter* from clinical and environmental samples. The amplification was based on *hip*O and 23S rDNA genes from *C. jejuni*, *gly*A gene from *C. coli*. *C. lari* and *C. upsaliensis* and *sap*B2 gene from *C. fetus* subsp. *fetus*. The detection of products from multiplex PCR has moved on from separation of amplified fragments by gel electrophoresis to real time PCR where, through the use of labelled probes, a fluorescent signal is detected directly during the amplification. In addition of being a rapid, sensitive and effective method, it has the advantage of quantification as well as identification and can be used directly without a need for culture. *Campylobacter jejuni* and *C. coli* were successfully detected and discriminated from each other in faecal, feed and environmental samples (Leblanc-Maridor *et al.*, 2011).

1.1.6 Fine Discrimination Between *Campylobacter* Strains

Historically many systems (phenotypic methods) have been proposed to differentiate between *Campylobacter* strains including serotyping and phage typing. However, subtyping campylobacters and differentiation became considerably easier with the development of molecular techniques (genotyping methods), like: multilocus sequence typing (MLST; Dingle *et al.*, 2005), Amplified Fragment Length Polymorphism (AFLP; Lindstedt *et al.*, 2000) and *fla* typing (Meinersmann *et al.*, 1997; Müller *et al.*, 2011).Two recent reviews of *Campylobacter* typing methods cover the advantages and disadvantages of the most commonly used methods (Eberle and Kiess 2012; Clark *et al.*, 2012).

Serotyping was one of the major methods used for *Campylobacter* strain discrimination before being superseded by molecular techniques. It is not widely used now, as many laboratories do not have the expertise or the antisera required for performing the assays (On *et al.*, 2008). The system is based on the detection of the surface antigens and the differences between them (Wiedmann, 2002) using different antisera. The first serotyping method performed by Berg *et al.* (1971) placed campylobacters into three serotypes on the basis of their heat stable antigens. Later methods include the Penner and Hennessy (1980) system and Lior *et al.* (1982) system, were based on the identification of heat stable and heat labile antigens respectively.

Phage typing is another phenotypic method that has been applied to discriminate between strains of *Campylobacter* (On *et al.*, 2008); however, this was superseded by molecular methods. Susceptibility to selected phages is

used for such discrimination. It was used in conjunction with serotyping to give better discrimination (Frost *et al.*, 1999). The technique involves inoculating a bacterial lawn, made from the strain to be tested, with different phages and examines their reaction pattern to these reference phages (Grajewski *et al.*, 1985). This scheme was described by Frost *et al.* (1999) where the individual *Campylobacter* phage types were defined as two or more different *Campylobacter* isolates giving the same phage reaction pattern. Different schemes were described worldwide including in the United States (Grajewski *et al.*, 1985), United Kingdom (Salama *et al.*, 1990) and Canada (Khakhria and Lior, 1992) but they shared some common phages.

Compared to the phenotypic methods, genotyping methods are more sensitive and can provide higher levels of differentiation, reproducibility, standardization and typeability (Wassenaar and Newell, 2000; Wiedmann, 2002). MLST has become a favoured genotyping method with the advantage of precision and differentiation by directly sequencing the amplified DNA of a specific set of genes especially those with housekeeping function (On *et al.*, 2008). This method was introduced by Maiden *et al.* (1998) in order to compare typing results between different laboratories. Dingle *et al.* (2001) developed this method to differentiate *C. jejuni* strains after which Miller *et al.* (2005) extended this method for *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*. It has the advantage of reproducibility, ease of interpretation, yet it can be relatively expensive (Lévesque *et al.*, 2008).

Amplified fragment length polymorphism (AFLP) is a method used to fingerprint *C. jejuni* subsp *jejuni* strain and does not require a previous

knowledge of genomic DNA sequence. This method was developed by Vos *et al.* (1995). It involves amplification of DNA fragments that were initially digested with two restriction enzymes and ligated to nucleotide adaptors that serve as a template on which primers anneal. Following electrophoresis, the fragments produced are detected using sequencer or by a fragment analyzer (On *et al.*, 2008) to produce a fingerprint, unique to the strain tested. Forty distinct patterns of ninety one *C. jejuni* strains were distinguished using this method (Lindstedt *et al.*, 2000).

The sequences of the *Campylobacter* flagella genes *fla*A and *fla*B can be used as a basis for discrimination in two different ways (*fla* typing methods). The first one is fla-RFLP which combines PCR amplification with RFLP (restriction fragment length polymorphisms), where the PCR amplicons are digested, electrophoresed and documented using software to identify the RFLP profiles. This method is simple, reliable and useful for subtyping *C. jejuni* and *C. coli* strains. It was successfully used for typing 26 *Campylobacter* strains from nine laying hens and resulted in the discrimination of 13 *C. jejuni* and 13 *C. coli* strains (Müller *et al.*, 2011). To overcome problems associated with reproducibility and variations between different laboratories, another way of *fla* typing method was developed which uses the *fla*-SVR (short variable region). Short variable regions were identified in the sequenced *fla*A gene and used as a template on which primers were designed for amplification. Following electrophoresis, the fragments are sequenced and compared to the reference SVR from data bases available (Meinersmann, *et al.*, 1997).

1.1.7 Growth and Survival of C. *jejuni* and *C. coli*

Campylobacter jejuni and *C. coli* are thermophilic with 42 °C thought to be their optimum (Park, 2002). These strains do not grow at temperatures of 55 °C or above. For that reason, Levin (2007) suggested that these organisms should be described as thermotolerant rather than thermophilic. *Campylobacter jejuni* and *C. coli* are sensitive to NaCl concentrations of 2 % and above (Park, 2002) with, 0.5 % NaCl thought to be optimum for their growth. The optimum pH for *Campylobacter* growth is between 6.5 to 7.5 with pH 4.9 thought to be the lowest and pH 9.0 to be the highest that strains of C. *jejuni* and *C. coli* can tolerate (Silva *et al.*, 2011).

Although large numbers of studies have been performed on *Campylobacter* survival they cannot be easily compared with each other as they are done under different conditions. For example survival at a particular temperature will be influenced by the atmosphere provided and by the medium in which the bacteria are heated, whilst the enumeration method, often using antibiotic containing selective agar, may significantly affect recovery of damaged cells. However it is clear that campylobacters can be easily inactivated by heat treatments. They were inactivated completely from ground beef cooked in the oven at 190 and 218 °C when the internal meat temperature reached 70 °C. Similar inactivation was achieved at both temperatures, yet at 218 °C the meat internal temperature increased faster (Stern and Kotula, 1982). Freezing and thawing reduces *Campylobacter* spp. populations (Stern and Kazmi, 1989). However, freezing does not eliminate campylobacters from contaminated foods (Lee *et al.*, 1998) and can act to preserve viability. It was reported that freezing

ground beef at -15 °C resulted in the reduction of *Campylobacter* cell numbers within three days, followed by stabilization of numbers, until the end of the incubation period (Stern and Kotula, 1982).

1.1.8 *Campylobacter* Sources

Chickens are widely believed to be the main source of campylobacters that cause human disease; however, they are not the only source. Other sources include: turkeys, ducks, lambs (Humphrey *et al.,* 2007), rodents, insects (Newell and Fearnley, 2003), cattle (Stanley and Jones, 2003), sheep (Scott *et al.,* 2012), pigs (Nielsen *et al.,* 1997; Harvey *et al.,* 1999) and other avian species. The drinking of untreated water or rain water (Nygard *et al.,* 2004), ground water (Hanninen *et al.,* 1998), and raw milk (Blaser *et al.,* 1979) can be sources of these bacteria. Lastly, campylobacters can be isolated from shellfish if they were grown in *Campylobacter*-contaminated waters (Wilson and Moore, 1996).

1.1.9 *Campylobacter* in Poultry

It was reported by Meldrum *et al.* (2006) that the incidence of campylobacters in whole raw retail poultry meat in the UK was high, with the number of birds testing positive being 68-73.1% in Wales, 83.3% in England and 57% in Northern Ireland.

A recent European Food Safety Authority survey indicated that campylobacters were present in more than 80 % of UK broiler chickens in common with many member states but with the exception of certain Scandinavian countries where

the incidence was low (Norway 5.1 %,, Sweden13.2 % and Denmark 16.5 %). On the other hand the incidence in Finland showed a marked difference between the first and second halves of the year, where the incidences were 1.8 % during January to May and 6 % through June to October (EFSA, 2011). *Campylobacter* colonization levels of individual chickens is high and falls within the region of 5 to 9 $\log_{10}$ CFU g^{-1} of the caecum content (Rudi *et al.,* 2004; Atterbury *et al.,* 2005). Colonization occurs widely in the avian intestine with the caeca, large intestine and cloaca carrying notably high populations of campylobacters (Beery *et al.*, 1988; Chaveerach *et al.*, 2004). Seven *Campylobacter* species and two subspecies have been isolated from commercial chickens in Ireland. Those include *C. coli*, *C. concisus*, *C. fetus*, *C. hyoilei*, *C. jejuni* sub *jejuni*, *C. jejuni* sub *doylei*, *C. lari* and *C. mucosalis* (Lynch *et al.*, 2011).

Campylobacters are part or the normal intestinal flora of chickens, yet previous studies reported the role of *C. jejuni* in the development of focal hepatitis in chickens (Peckham, 1958 cited in Jennings *et al.,* 2011). It is a disease characterized by the presence of focal lesions in the liver that are 1-2 mm in size and greyish to whitish in colour (Burch, 2005). Liver from diseased and normal commercial chickens was examined and it was found that campylobacters were present in both of them but those with signs of focal hepatitis had higher rate of isolation. Failure to replicate the disease in healthy chickens using campylobacters isolated from diseased chicken, indicates that unknown factors are involved in its development (Jennings *et al.,* 2011).

1.1.10 *Campylobacter* Transmission in Poultry

1.1.10.1 Vertical Transmission

It was suggested that *Campylobacter* could be transmitted to flocks through contaminated eggs, but this has not been conclusively proven (Callicott *et al.*, 2006), thus there is a debate about this way of transmission. Many strains have been isolated from the reproductive tract of poultry including the oviduct (Buhr *et al.*, 2002), but it is still unknown whether the strains isolated have caused embryo colonization or not.

In an experiment performed by Allen and Griffiths (2001) to assess egg shell colonization and penetration, it was found that egg shells are permeable to *C. jejuni* when immersed in a strain suspension, thus eggs can be contaminated by any contact with faeces. With such a contamination method, the bacteria were found in the inner shell or membranes but not in the egg content (Neill *et al.*, 1985), which may only be contaminated when in contact with faeces via shell cracks (Doyle, 1984). If vertical transmission were to occur, campylobacters would be expected to be detected immediately after hatching from *Campylobacter* colonized parent flocks; however this seldom happens with first detection usually occurring at around 3 weeks (Newell and Fearnley, 2003).

In an experiment performed by Cox and others (2009), it was found that *C. jejuni* can disseminate to the lymphoid organs of day-old broiler chicks and remain there for an extended time after oral or intra-cloacal inoculation. In addition, campylobacters were isolated from eggs and hatchery fluff which

may indicate the possibility of this route of transmission. On the other hand, in around 60,000 progeny parent breeders, hatched from eggs that their parent flocks were positive for campylobacters, no evidence was found for any vertical transmission (Callicot *et al.*, 2006).

1.1.10.2 Horizontal Transmission

Horizontal transmission of campylobacters is the most likely way in which poultry flocks become infected. Once the pathogen is transmitted, it is difficult to eliminate. Although broiler chickens normally live in a closed environment in barns, campylobacters are ubiquitous in the environments around the broiler houses, which could result in flock contamination when they enter the houses (Stern *et al.*, 2001). They can colonize chickens in several ways, such as consumption of contaminated feeds, or through contaminated broiler house equipment, boots (Wingstrand *et al.*, 2006; Lindqvist and Lindblad, 2008) or coverall of personnel (Humphrey *et al.*, 2007), or through the transfer of excreta from one batch to the next, or from consumption of contaminated water (Hald *et al.*, 2001).

For campylobacters to colonize an entire flock in a short time, it needs only one infected bird (Newell and Fearnley, 2003). Several studies on the prevalence of *Campylobacter* in broiler flocks reported that flock infection depends on various factors that include: implementation of biosecurity measures in farms, inadequate disinfection between chick placements, multi-unit sites, flock thinning (partial depopulation of the flock), the presence of other animals on the farm, the presence of rodents, seasonal insects (Berndtson

et al., 1996, Newell and Fearnley 2003; Bouwknegt *et al.,* 2004; EFSA 2007) and the season. Wills and Murray (1997) performed a study to find out the variations in temperatures in different seasons and they found that in warmer months (May to October), the percentage of campylobacters in chicken was high (87-97 %), whereas during months of lower temperatures in December and January, the percentages were 7 % and 33 %, respectively.

The age of the broiler plays a role in colonization. Three week old birds are more susceptible than one to two weeks old birds, and this is thought to be due to maternally derived, circulating, anti-*Campylobacter*, immunoglobulin Y antibodies which decline after hatching (Sahin *et al.*, 2003; Cawthraw and Newell, 2010). Cawthraw and Newell (2010) also reported that, the reasons for increased susceptibility between 0 and 3 days after hatching, followed by reduced susceptibility until around three weeks in unknown.

1.1.11 Contamination of Poultry Meat

Contamination of poultry meat from *Campylobacter* colonised birds occurs during slaughter and processing steps such as defeathering, evisceration and the cooling of carcasses (Kramer *et al.,* 2000; Keener *et al.,* 2004; Reich *et al.,* 2008). The content of the intestinal tract maybe transferred to the surface of the skin if the intestines leak or rupture during processing where the contents may remain in the cervices and channels (Chantarapanont *et al.,* 2003). Campylobacters will persist on the surface of the poultry meat until the conditions are favourable for their growth and multiplication.

1.1.12 *Campylobacter* Food-Borne Disease

Campylobacter jejuni has been found to be a major cause of food-borne illness (gastroenteritis) in many parts of the world (Griffiths and Park, 1990; Blaser, 1997; Friedman *et al.*, 2000), including the United Kingdom (Friedman *et al.*, 2000; Newell *et al.*, 2010). Campylobacters were known as disease causative, in animals since 1909, but were not known to be involved in human diseases until about 1980 (Vandamme, 2000).

Campylobacter spp. can lead to a number of sequelae including: reactive arthritis (Overell and Willison 2005; Ternhag *et al.*, 2008), which is a sterile auto-immune post-infectious process that affects some joints (Peterson, 1994; Allos and Blaser 1995); Guillain-Barré syndrome (Nachamkin, 2002), which is autoimmune demyelinating disorder of the peripheral nervous system leading to symmetrical weakness of the limbs, respiratory muscles and loss of reflexes, that may become chronic or lead to death in the acute phase (Mishu *et al.*, 1993; Mishu and Blaser 1993; Allos, 1997) and Miller Fisher syndrome which is a variant of Guillain-Barré syndrome (Othsuka *et al.*, 1988). Other sequelae include meningoencephalitis and encephalopathy but these are rare (Van der Kruijk *et al.*, 1994). Recently, *C. jejuni* was reported to be responsible for causing acute encephalopathy in a young female patient with symptoms of fever, headache, abdominal cramps, watery diarrhoea and rapid onset of neurological deterioration (Lepur *et al.*, 2012). Spondylodiscitis is caused by inflammation of the intervertebral disc space and the area adjacent to it and has been associated with *C. fetus* infection and latterly *C. jejuni* has also been implicated (Tappe *et al.*, 2012). Recently, *C. concisus* which is known to cause

chronic intestinal diseases (Kaakoush *et al.*, 2011) was found in faecal material from patients suffering with Crohn's disease (Man *et al.*, 2010; Kaakoush *et al.*, 2011) which is a serious inflammatory bowel condition. It is important to understand that campylobacters are not the only pathogens present in the intestines. Thus, they are not necessarily the cause of this disease (see van Heel *et al.*, 2001) and their roles as causative agents, if any, need to be investigated.

Reports by the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) within the last five years indicate that campylobacteriosis is the most frequently reported food-borne bacterial disease in Europe followed by salmonellosis and yersiniosis (EFSA, 2007). The infection rate in developed countries has been estimated to be between 20 and 60 people per 100,000 per annum (Acheson, 2001). *Campylobacter* infection is the main cause of bacterial enteritis in England and Wales. More than 65,000 cases were reported in the United Kingdom with 195,000 in the European Union in 2009 (EFSA, 2011). Since many cases are not reported, these numbers are almost certainly an under-estimate of the disease burden, with the underlying figure estimated to be 450,000 in the UK (Strachan and Forbes, 2010). Nichols and colleagues (2012) estimated that there are about 500,000 cases in the UK every year with 80,000 general practitioner (GP) consultations. The infection rate increased in older people over 50 in the years between 2004 and 2010 probably because of the increased use of drugs which makes them more susceptible than other age groups. Reports of *Campylobacter* infecting humans have been increasing all over the world (Frost *et al.*, 2002). It was reported that campylobacters result in illness in about 1 % of the

population with about 13,000 hospitalized cases and more than 100 deaths yearly in the USA (Anonymous, 2007). Although campylobacteriosis is known to be primarily a sporadic disease (Phillips, 1995) with few affected people and the source of infection is not known, (poultry is most likely), outbreaks involving large numbers of cases can occur when campylobacters contaminate the water supply (Blaser, 1997) or bulk milk containers (Heuvelink *et al.*, 2009).

It is believed that humans become infected largely through eating contaminated or undercooked poultry (Istre *et al.*, 1984; Kapperud *et al.*, 1992; Evans *et al.*, 1998; Newell and Fearnley, 2003) or through the cross contamination of other foods while preparing poultry (Jacobs-Reitsma, 2000). Studies have indicated that the presence of campylobacters in a high percentage of poultry and their products is the major cause of *Campylobacter* infections (Stoyanchev *et al.*, 2007). When contaminated poultry meat was implicated as the source of campylobacteriosis, 20-30 % of the cases resulted from the way these broilers were handled, prepared and consumed. Between 50 and 80 % of the MLST subtypes from human disease cases are typical of the chicken reservoir as whole (EFSA, 2010).

Campylobacter jejuni was reported to be responsible for more than 12 times the number of cases of human campylobacteriosis as that of *C. coli* in a surveillance held in England and Wales (Friedman *et al.*, 2000), but *C. coli* remains a significant cause of such disease. *Campylobacter jejuni* was reported to be responsible for 90 % of human campylobacteriosis (Kramer *et al.*, 2000). Tam *et al.* (2003) reported that *C. coli* was responsible for over than 25, 000

cases of the disease in England and Wales in 2000. Other species have been reported to be involved in diarrhoeal illness in humans including; *C. upsaliensis*, *C. concisus*, *C. gracilis*, *C. hyointestinalis*, and *C. curvus* (Lindblom *et al.* 1995; Gorkiewicz *et al.*, 2002; Labarca *et al.*, 2002; Lastovica and Leroux, 2003; Maher *et al.*, 2003; Boyanova *et al.*, 2004) and *C. lari* (Griffiths and Park, 1990). A recent study in Ireland revealed that, of 329 clinical samples confirmed as being *Campylobacter* species: 66 % were *C. jejuni*, 6.7 % were *C. coli* , 2.4 % were *C. fetus*, 1.2 % were *C. upsaliensis*, 1.5 % were *C. hyointestinalis*, 0.6 % were *C. lari* and 24.4 % were *C. ureolyticus* (Bullman *et al.*, 2011).

Campylobacter related disease is generally diagnosed using cultural methods to isolate the bacteria. The use of non-cultural techniques such as antigen detection or PCR for detection of different pathogens from stool samples is increasing (M'ikanatha *et al.*, 2012; Stigi *et al.*, 2012). Yet replacing cultural methods is disadvantageous as no isolates will be available for further testing (for example subtyping) making epidemiological studies of outbreaks difficult (Jones and Gerner-Smidt, 2012). Culturing samples and forwarding them to public health laboratories remains the recommended procedure by the Center for Disease Control and Prevention in the USA (Gould *et al.*, 2009).

1.1.13 Pathogenesis of *Campylobacter jejuni*

Campylobacter has evolved specific mechanisms to survive within host cells (Watson and Galán, 2008) but these processes are not well understood probably due to the lack of similarities between campylobacters and other pathogens (Guerry, 2007). The major pathogenic mechanisms of *Campylobacter* involve flagella proteins, colonization factors, adherence proteins, invasion proteins and toxin production (Dasti *et al.*, 2010).

Studies have demonstrated the role of flagella and motility in invasion and pathogenesis (Wassenaar *et al.*, 1991; Golden and Acheson, 2002; Carillo *et al.*, 2004), where flagellar mutants that affect motility can completely eliminate bacterial internalisation. Motility is required for intestinal colonization (Barreto-Tobon and Hendrixson, 2012) of poultry and humans (Wosten *et al.*, 2004). Moreover, it is used in the interaction with and invasion of human intestinal epithelial cells (Wassenaar *et al.*, 1991). The flagella of *C. coli* and C. *jejuni* consist of major (FlaA) and minor (FlaB) flagellins (Guerry, 2007). The FlaA flagellin plays a role in the processes of adherence and colonization of the gastrointestinal tract as well as host invasion (Jain *et al.*, 2008). The gene encoding FlaA is regulated by a σ^{28} promoter whilst the FlaB flagellin gene is regulated by σ^{54} (Jagannathan and Penn, 2005). Both *C. jejuni* and *C. coli* have polar flagella (either a single one or one at each end) that are composed of O-linked glycosylated flagellins and this glycosylation is thought to play a role in facilitating the approach to the attachment sites on intestinal epithelial cells (Dasti, *et al.*, 2010). Campylobacters use a flagellar based type III secretion system that is responsible for secreting and delivering proteins

needed for invasion and affecting host cell function, such as the Cia protein group (Barreto-Tobon and Hendrixson, 2012).

Perhaps the best characterized of the *C. jejuni* adhesins are the 37 kDa outer membrane protein termed CadF (*Campylobacter* adhesion to fibronectin) (Konkel *et al.*, 1999), a 42.3 kDa surface exposed lipoprotein termed JlpA (Jin *et al.*, 2001) and a 28 kDa periplasmic membrane-associated protein termed PEB1 (Pei and Blaser, 1993).

Surface components of the outer membrane paly a role in the interaction between campylobacters and their hosts. Examples of these includes the surface polysaccharide (lipopolysaccharides and lipooligosaccharides) which contribute to internalization indirectly by promoting bacterial attachment to host cells (Hu and Kopecko, 2008).In addition to that, lipid rafts, which are microdomains within plasma membranes enriched with cholesterol and glycolipids, appear to have a role in efficient *Campylobacter* entry to the host cell (Watson and Galán, 2008).

Campylobacters, while adhering to or invading host cells, secrete proteins, toxins and other molecules that adversely affect host cells. These effectors stimulate or inhibit host signal transduction pathways, promote cytokine production, trigger inflammation, change host cell mitogenesis, cause host cell death and water malabsorption (Hu and Kopecko, 2008). Cia proteins are required for invasion as evidenced by the inability of mutants to invade or survive within eukaryotic cells (Barreto-Tobon and Hendrixson, 2012). However, the only verified *Campylobacter* toxin identified is the cytolethal

distending toxin (CDT), which is produced by a number of *Campylobacter* species including *C. jejuni, C. coli, C. fetus, C. lari* and *C. upsaliensis* (Dasti *et al.*, 2010). It is suggested that this toxin is the key factor for campylobacteriosis, yet mutated strains that do not express this protein can still cause the disease (Abuoun, *et al.*, 2005) but reports suggest the invasion ability was decreased 10-fold (Biswas *et al.*, 2000). CDT is composed of three subunits; CdtA, CdtC and the active subunit CdtB. The role of CdtA and CdtC needs to be investigated (Ceelen *et al.*, 2006) yet they are required for the delivery of CdtB to the host cell. CdtB is responsible for binding to the cell membrane (Lara-Tejero and Galan, 2001), where its active subunit exhibits DNaseI-like activity that causes host cell damage (Ge *et al.*, 2008).

1.1.14 Antibiotic resistance

Antibiotics are used in culture media as selective agents. Yet, not all species can be isolated by the use of a single medium. A number of antibiotics often used in the isolation media were tested on different strains either singly or in combination, where differences in their sensitivity and resistance were noted between them (Corry *et al.*, 1995b).

The β-lactam antibiotics, such as cefoperazone, are the most often used antimicrobials in *Campylobacter* isolation media (Silley, 2003). Resistance to this class of antibiotics can be controlled through the intrinsic resistance and the production of β-lactamase (Li *et al.*, 2007). Due to cephalothin susceptibility, some strains can be missed in the sample (Brooks *et al.*, 1986)

such as: *C. upsaliensis* as antibiotic selection does not support its isolation (Warmsley and Karmali, 1989).

In addition, Campylobacters are resistant to a number of antibiotics including; bacitracin, novobiocin, rifampin, streptogramin B, trimethoprim and vancomycin (Corry *et al.,* 1995b). Their resistance mechanism is not clear, but Zhang and Plummer (2008) reported that it could be related to their low permeability to the *Campylobacter* cell membrane and active efflux that is conferred by the multidrug-efflux transporters.

Some antibiotics are used in the treatment of campylobacteriosis, which include fluoroquilonones (e.g., ciprofloxacin) and macrolides (e.g., erythromycin). Development of resistance to antibiotics by campylobacters is of concern to human health.

It was reported that 10-47 % of campylobacters from human sources are resistant to ciprofloxacin (Gaudreau and Gilbert, 2003). Resistance to fluoroquinolones is mediated by point mutations in quinolone resistance determining region in DNA gyrase A (Zhang and Plummer, 2008) as well as the efflux pump CmeABC, which works by reducing antibiotics accumulation in *Campylobacter* cells (Luo *et al.,* 2003).

Resistance to macrolides is associated with target modification and active efflux (Cagliero *et al.,* 2006; Mamelli *et al.,* 2005). It was reported that in Africa, campylobacters from human sources are highly resistant to erythromycin, whereas those from food producing animals show low resistance

levels (Gibreel and Taylor, 2006). Studies from Asia are rather limited because there are few surveillance labs, but by combining limited data from Japan, Thailand and Korea, Luangtongkum *et al.* (2009) estimated that in Asia, around 5 % of *C. jejuni* and 14-62 % *C .coli* from humans, swine, broilers and cattle were resistant to macrolides.

A number of studies of poultry isolates have been performed to determine their contribution to antibiotic resistance. In a study performed in Québec to determine antibiotic resistance in organically raised chickens, it was reported that *C. jejuni* were resistant to tetracycline, erythromycin, azithromycin and clindamycin and sensitive to chloroamphenicol, ciprofloxacin, gentamicin, nalidixic acid and ampicillin (Thibodeau *et al.,* 2011). In another study performed in Japan on the susceptibility of campylobacters from broilers to eight antimicrobials, it was reported that campylobacters are sensitive to gentamicin, chloroamphenicol and erythromycin but resistant to dihydrostreptomycin, aminobenzyl penicillin, enrofloxacin, oxytetracycline and nalidixic acid (Haruna *et al.,* 2012).

For food safety and public health, where flouroquinolones are used in campylobacteriosis treatments, *Camplylobacter* resistance to these antibiotics results in losing their efficiency and makes the use of them more difficult (Luangtongkum *et al.,* 2009).

1.1.15 Control of Campylobacters

In order to reduce the unacceptably high levels of human *Campylobacter* infection, a number of suggestions for reducing the levels of *Campylobacter* in food have been proposed. In selecting an appropriate method consideration should be given whether the method would change the organoleptic or nutritional value of the food. Also it should be safe, cheap and acceptable to the public (Corry *et al.*, 1995a).

Rosenquist *et al.* (2003), reported that mathematical models of risk indicated that reducing exposure to *Campylobacter,* reducing their numbers on chicken carcasses by 2 $\log_{10}$ CFU/g, would reduce the numbers of people who get campylobacteriosis by 30-fold.

Campylobacters are sensitive to drying, low humidity, freezing and freeze thaw stress and oxygen. For their control, these points should be considered when putting strategies in place (Silva *et al.,* 2011). There are many suggested methods for the reduction of *Campylobacter* numbers in poultry meat, starting with prevention of infection of the birds, reduction of contamination during processing and treatment of the final meat product.

Any action that reduces the horizontal transmission (1.1.10.2) of *Campylobacter* is considered to be effective in reducing the chance of *Campylobacter* contamination. The possible ways for poultry meat contamination in the slaughter houses are discussed in section 1.1.11. In order to reduce or prevent this contamination, it is important to separate the positive

from negative flocks in the slaughter house (Wagenaar *et al.*, 2006). Such approach was taken in Denmark by the use of a rapid testing protocol (4h gel based PCR technique) for differentiating the positive and the negative flocks (Krause *et al.*, 2006) at slaughter. In addition to that, in Denmark, insect nets were used and successfully reduced *Campylobacter* introduction into broiler houses (Hald *et al.*, 2007), yet this type of control has not been investigated in other countries (Wassenaar, 2011).

Campylobacters can survive well on surfaces within slaughter houses, on the equipment (Peyrat *et al.*, 2008) and on cutting boards (Wanyenya *et al.*, 2004). Gutiérrez-Martín and others (2011) evaluated a number of disinfectants against campylobacters using a suspension test which was not used before for determining their susceptibility. They found that compounds based on quaternary ammonium structures showed the highest levels of reduction (>6 $\log_{10}$ CFU) for all strains tested under all conditions. The sanitizing agents included chloramine-T, povidone-iodine, cetylpiridinium chloride, ethanol, isopropanol, chlorhexidine digluconate, formaldehyde and phenol.

Poor hygiene in the kitchen or cross-contamination from knives, utensils, cutting boards to food should be avoided to reduce risk of campylobacteriosis, which is thought to be a more common cause than eating undercooked meat (Luber, 2009). Chicken livers are excellent reservoir for campylobacters and should be cooked properly prior to eating (Whyte *et al.*, 2006).

Some studies have pointed out that consumption of certain foods correlates with a reduction in the risk of *Campylobacter* infection. Examples of these

foods include: poultry, red meat, vegetables and fruits (Rodrigues *et al.,* 2001; Neimann 2001). The reasons for this obvious discrepancy with regard to poultry and meat are not known. Also, Kapperud *et al.* (2003) stated that eating mutton and fish are protective factors against *Campylobacter* infection. These studies support the general consensus that eating what are considered to be healthy foods, can protect from diseases possibly because some fruits and vegetables contain antioxidants and carotenoids which will inhibit bacterial growth (Taylor and Engberg, 1999). However, since the consumption of chicken is also a risk factor, the frequency of consumption is an important consideration as with frequent consumption, the immune system will be stimulated and will prevent infection.

Antimicrobial methods available can be physical, chemical or biological. Trials of these methods have produced mixed results (Newell and Wagenaar, 2000) without a single treatment being successful. Furthermore some treatments might result in the selection of treatment-resistant survivors with increased virulence rendering further treatment ineffective; thus, leading to the conclusion that a combination of methods should be considered.

The use of chemicals such as chlorine, trisodium phosphate, ozone and organic acids in carcass washing is effective in microbial reduction (James and James, 1997), however their use is not recommended as chemical residues might remain on the meat, consumers do not like foods containing additives and there are problems in disposing of the chemicals (James *et al.*, 2007). On the other hand, the use of physical methods such as heat and cold cycles has the advantage that the likelihood of resistance development is low compared with

chemical treatments (organic acids, essential oils) but they may change the texture and composition of the food, which would make them less desirable to the consumer. James *et al.* (2007) reported that rapid heating above 70 °C should be followed by rapid cooling to prevent muscle heating and protein denaturation in chicken meat.

Washing the contaminated surfaces of equipment with hot water and hypochlorite was significant in reducing *Campylobacter* numbers. Dipping or spraying poultry carcasses or poultry parts with lactic or citric acid or hypochlorite can result in 1.0-1.5 $\log_{10}$ count reduction (Ellebroek *et al.*, 2007). Freezing the carcasses was reported to be effective in *Campylobacter* reduction at -20 °C (Lee *et al.*, 1998). In addition, James *et al.* (2007) reported that the use of steam or hot water combined with chilling or crust freezing can produce greater reduction than using the steam or hot water alone. On the other hand some pathogens can survive freezing for long periods (Lund, 2000), and therefore combination treatments could provide a greater hurdle causing cumulative damage, for example by freezing and thawing and heating in combination (Mackey, 2000). In USA, deep or super chilling of chickens at temperatures near its freezing (-1 to -2 °C) is used (Jul, 1986).

The use of antibiotics is not acceptable because it results in the development of antibiotic resistant strains, which may lead to reduced efficiency of human disease treatment (Hariharan *et al.*, 2004). Competitive exclusion (the Nurmi principle) is another strategy that is used to reduce intestinal colonization by pathogenic microorganisms. It has proved effective for *Salmonella* control but

not for campylobacters (Mead, 2002) as it does not eliminate them completely, (Lin, 2009).

Biological treatments have been developed for pathogen control. Examples of these include the use of phage, (covered in next section) and bacteriocins (Sirsat *et al.,* 2009). The application of bacteriocin producing bacteria such as *Paenibacillus polymyxa* or bacteriocins into chicken feed has been shown to be effective in controlling campylobacters, but this needs further investigation (Stern *et al.,* 2005). *Lactobacillus salivarius* isolated from a Tunisian chicken caecum has shown antimicrobial activity against campylobacters (Kergourlay *et al.,* 2012). Plant extracts to control campylobacters were used in a study where Chinese leek extracts were shown to inhibit *Campylobacter* growth (Lee *et al.*, 2004). More recently, Castillo *et al.* (2011) reported the use of extracts from edible and medicinal plants for the same purpose. As these are natural plant extracts, they can be considered as good candidates for food preservation and pathogen control.

1.2 Bacteriophages

1.2.1 Introduction

Bacteriophages, often shortened to 'phages', are viral parasites of bacteria which exist universally in the environment. The total number of phages on the planet has been estimated to be 10^{31} phage particles and thus they are the most numerous biological entity on earth (Hendrix *et al.*, 1999). They can be found in sea water, soil and in the intestines of animals, where they have the ability to infect, multiply and kill their host bacteria, including *Campylobacter.*

1.2.2 Bacteriophage Life Cycles

There are two types of phage life cycle, lytic and temperate. The lytic phages life cycle is simple and has no intermediate form. It starts with adsorption of the phage to the surface of the bacterial cell, followed by injection of the nucleic acid through the cell wall. After that, phage nucleic acid and protein capsids are synthesized using the host cell's biosynthetic apparatus and phage particles are assembled. Finally, the bacterial cell envelope ruptures to release the phage progeny. Temperate phages have a different life cycle which can follow two routes. The first two steps (adsorption and injection) are the same as for lytic phages, then the temperate phage will either continue replication and lysis in the same way as lytic phages or the nucleic acid will integrate into the host chromosome. When excised, the temperate phage may take part of the host DNA with it and continue infecting and integrating into a new host where it may add the additional excised DNA to the host chromosome (Sulakvelidze *et al.*, 2001). These events may enable the horizontal transfer of genes that can include virulence factors or antimicrobial resistance determinants in a process known as transduction. In contrast to both lytic and lysogenic phages, the filamentous phages, for example M13, infect the bacteria without killing the host. Instead, they result in continuous excretion of viral particles (Harper and Kutter, 2008).

1.2.3 The History of Bacteriophages

In 1896, Ernest Hankin, a British bacteriologist made the first report of phage when he found that the water of the Ganges river in India had an antibacterial effect against *Vibrio cholera* (Hankin, 1896; cited in Connerton and Connerton, 2006). After that Frederick Twort (1915) and Felix d'Herelle (1917) discovered that phages are viruses (d'Herelle, 1922; cited in Connerton and Connerton, 2006). Frederick Twort described a transmissible "glassy transformation" of micrococcus culture, which resulted in the degradation of bacteria (Connerton and Connerton, 2006). Two years later, microscopic organisms were reported by d'Herelle (1917; cited in Connerton and Connerton, 2006). These organisms were able to lyse *Shigella* cultures on plates resulting in a clear zone called a plaque. He introduced the term phage within the same year and suggested that such phage could be used to reduce or stop bacterial infection.

In 1917, d'Herelle used phages which were isolated from poultry excreta to cure chicken typhus and dysentery in humans (cited in Summers, 2001) which are caused by *Rickettsia* spp. and *Shigella* spp. respectively. Subsequently, in 1921, a skin disease caused by *Staphylococcus* spp. was treated by applying phages (Bruynoghe and Maisin, 1921).

1.2.4 Bacteriophage Structure and Taxonomy

Phages are composed of capsid proteins that encase nucleic acid (Casjens, 2005). Phage classification evolved gradually from their first description by d'Herelle, who classified them as a single species with many races. Holmes (1948; cited in Ackermann, 2003) classified phages as a suborder, with a single

family and a single genus under one order, the *Virales*. This system was premature and was based on the symptoms of the diseases of their hosts, thus it was forgotten. Later, Lwoff, Horne and Tournier (1962; cited in Ackermann, 2003) published a system for their classification according to their morphology and nucleic acid type. They recognised six types of phages which were tailed phages, filamentous phages and cubic phages with single-stranded or double-stranded DNA (Ackermann, 2003). This system formed the basis for the present phage classification.

Phages are also classified based on the type of the genetic material, whether they have single or double stranded DNA or RNA and can be further subdivided according to their morphology. The major group of double stranded DNA phages are tailed and classified together under one order with three families (Proux *et al.*, 2002). According to the International Committee for Taxonomy of Viruses (ICTV), viruses are classified into three orders including 61 families and 241 genera. Among them, phages belong to one order (Caudovirales), with 13 families and 30 genera (Ackermann, 2003).

More than 5100 phages have been examined since 1959. When examined under the electron microscope, 96 % of the phages were found to be tailed and 4 % had polyhedral, pleomorphic or filamentous morphologies (Ackermann, 2007). Tailed phages were found to contain linear double-stranded DNA genomes of different sizes ranging from 18 to 500 kb. These virions are composed of a head that is icosahedral in shape containing DNA and a tail responsible for binding to the host for the delivery of DNA into the host cells (Casjens, 2005). The three families of tailed phages are, the *Myoviridae*

(24.5 % of those examined; Ackermann, 2007) that include phages with contractile tails such as T4 phage, the *Siphoviridae* (61 % of those examined; Ackermann, 2007) that include phages with long non-contractile tails such as λ phage and the *Podoviridae* (14 % of those examined; Ackermann, 2007) that include phages with short tails such as T7 phage (Ackermann, 2007). Phages in the order *Caudovirales* share some physical properties but it is difficult to group them according to their DNA or protein sequences because of the diversity caused by horizontal gene transfer, the presence or absence of particular genes in all tailed phages that could be used as an indicator for their identification (Casjens, 2005) and the mosaic nature of phage genome (Hendrix *et al.*, 1999). Based on morphological observations of the phage reported to infect *Campylobacter* more than 170 belong to the family *Myoviridae* (Sails *et al.*, 1998), two belong to the *Siphoviridae* family and one to the *Podoviridae* (Connerton *et al.*, 2008).

Various alternative approaches have been proposed for phage classification. Such approaches include; construction of a phage proteomic tree depending on the protein sequences (Rohwer and Edwards, 2002). Another approach is the use of genomic types, whether they contain single stranded RNA or DNA for dividing phages, and further differentiation according to their physical characteristics (tailed or filamentous; Lawrence *et al.*, 2002). The current system has hierarchical classification where different phage genera and species are put under the family. Six genera (λ, T1, T5, L5, C2 and ψM) were accepted by the ICTV for the family *Siphoviridae* (Proux *et al.*, 2002), six genera (T4, P1, P2, Mu, SPO1 and ϕH) for *Myoviridae* and three genera (T7,

P22 and ϕ29) for *Podoviridae*. These genera can be used for the classification of other phages. Based on the morphology, DNA-DNA hybridization, nucleic acid sequencing and serology, about 250 species are recognised (Ackermann, 2003). Lastly the use of a modular approach has been adopted to identify functional units within the gene content. This approach uses DNA and protein sequence similarities to recognise exchangeable functional units (Proux *et al.*, 2002). Although these modules cover a fraction of all phage genes, phage groups can be distinguished by their different combination of modules which can be the basis for a higher level of classification. Representing the relationship within phage populations will be possible automatically and dynamically with the use of this scheme, as it will allow the rapid inclusion of newly sequenced phage genomes (Lima-Mendez *et al.*, 2008).

1.2.5 *Campylobacter* Bacteriophage

1.2.5.1 Campylobacter Bacteriophage Sources and Isolation

When *Campylobacter* phages were reported back in the 1960s, they were isolated from cattle and pigs where they were reported as lytic against *Vibrio coli* (probably *C. coli* using present nomenclature) and *V. fetus* (probably *C. fetus)* (Fletcher and Bertschinger 1964; Firehammer and Border 1968; Fletcher 1968; cited in Connerton and Connerton, 2006). *Campylobacter jejuni* phages were recovered in 1980s arising from strains isolated from aborted fetuses that were inducible lysogenic phage recovered after treatment with mitomycin C (Bryner *et al.*, 1982; cited in Connerton and Connerton, 2006), although the source might suggest the bacteria were actually *C. fetus*. More recently, many different *Campylobacter* phages have been isolated from

different sources such as broiler chickens (Connerton *et al.*, 2004), retail poultry (Atterbury *et al.*, 2003a), pig manure, sewage and slaughter house out-flow (Salama *et al.*, 1989).

To isolate the phages, the collected samples should be centrifuged to remove debris followed by filtration to ensure the removal of any remaining bacterial cells in the phage suspension (Atterbury *et al.*, 2005). After that, they should be applied to an appropriate *Campylobacter* host strain suspended in a soft agar lawn. Plaques become visible after incubation under appropriate conditions. However, as a relatively small volume of suspension can be applied to the bacterial lawn, phage may be missed if they occur in low numbers. To overcome this problem, an enrichment method to increase their numbers was devised in which *Campylobacter* cells were incubated with the sample for 18 h prior to detection (Carvalho *et al.*, 2010b).

1.2.5.2 Campylobacter Bacteriophage Classes and Characteristics

Some *Campylobacter* phages have been characterized and found to have double stranded DNA genomes that are reported as belonging to the families *Myoviridae* and *Siphoviridae* (Sails *et al.*, 1998). The phages described by Frost *et al.* (1999) were studied and characterized by Sails *et al.* (1998), and were found to have icosahedral heads and long contractile tails indicating them to belong to the family *Myoviridae.* Phages belonging to the *Siphoviridae* and *Podoviridae* families were described in Russian Federation reports, but few details are available regarding the characteristics of these phages (Connerton *et al.*, 2008).

One of the systems used for characterizing phages utilizes the genomic size obtained by the use of pulsed field gel electrophoresis (PFGE). This allowed *Campylobacter* phages used in the scheme of Frost *et al.* (1999), to be divided them into three groups based on their genomic size and head diameter. Group I included two phages with head diameter of 140.6 and 143.8 nm and genomic size of 320 kb. Group II included five phages with average head diameter of 99 nm and average genomic size of 184 kb. Lastly, nine phages were placed in group III with an average head size of 100 nm and average genome size of 138 kb (Sails *et al.*, 1998).

In our laboratory at the University of Nottingham, all the phages isolated from poultry fall in to groups II and III (Atterbury *et al.*, 2003a; Connerton *et al.*, 2004; El- Shibiny *et al.*, 2005; Loc Carrillo *et al.*, 2005), possibly because Group I phages are not common in the UK. Both Group II and III phages are lytic phages typical of the *Myoviridae* family (International Committee on the Taxonomy of Viruses, 2004). Lysogenic phages have been reported from *C. fetus* (Ritchie *et al.*, 1983; cited in Connerton and Connerton, 2006) and from *C. jejuni* isolated from aborted sheep fetuses (Bryner *et al.*, 1982; cited in Connerton and Connerton, 2006), although as noted above the latter source is questionable.

The same sixteen phages of Frost *et al.* (1999) were classified into four groups (A, B, C and D) by Coward *et al.* (2006) depending on their activities against spontaneous transposon and defined mutants of *C. jejuni*. A correlation was shown between the structural grouping of Sails *et al.* (1998) and the phenotyping grouping of Coward *et al.* (2006). Groups B (φ3, φ8, φ10, φ14

and φ15) and D (φ4, and φ12) which are associated with motility and can be flagellotrophic are related to structural groups II and I respectively, whereas those that can interact with the capsules; groups A (φ1, φ2, φ6, φ11, and φ16) and C (φ5, φ7, φ9 and φ13) are all in structural group III but the important bacterial factors in sensitivity to phages in group C are not known (Coward *et al.*, 2006).

1.2.6 Incidence of *Campylobacter* Bacteriophages in Poultry

Different studies have been performed worldwide to isolate *Campylobacter* phages from poultry samples. Few have attempted to establish the incidence of *Campylobacter* phage in such samples but amongst those, Atterbury *et al.* (2003a), isolated and characterized *Campylobacter* phages from chilled and frozen retail poultry portions in UK. Such phages were isolated from 11.3 % of the chilled samples whereas all the frozen samples tested were negative. Prior to freezing the birds are scalded at high temperatures and this together with subsequent freezing and thawing cycles may have inactivated the phage. Phages were found in samples only when their host strains were also found. The phages did not replicate on samples inoculated with them and stored under refrigerated retail storage conditions, probably because campylobacters themselves do not grow on food samples under such conditions.

In the year 2005 the incidence of *Campylobacter* phages in UK poultry was studied and determined to be around 20 %, of 205 broiler chicken caeca sampled from 22 farms (Atterbury *et al.*, 2005) that were slaughtered between 22 and 46 days of age. Campylobacters were isolated from 63 % of the samples

and all of them were *C. jejuni* with no *C. coli* isolated. *Campylobacter* counts were significantly lower in samples with phages than those without phages.

In another study performed on caeca from organic and free range birds selected and slaughtered every three to four days until 73 and 56 days of age respectively, the *Campylobacter* phage incidence was found to be 51 % from organic birds whereas their isolation from free range birds was infrequent where only one phage was isolated (El-Shibiny *et al.*, 2005). The *Campylobacter* isolation percentage was high from organic birds (68.5 %) and from free range birds (90 %). This high presence could be due to outdoor birds having greater exposure to the environment resulting in the presence of more types of campylobacters and their phages. Connerton *et al.* (2004) reported in a study performed on a naturally phage-infected broiler chicken barn that phages and their hosts could be transferred from one flock to another.

In Denmark, the isolation rate of *Campylobacter* phages from broilers and ducks (intestines and abattoir sewage) of the Danish poultry production was studied and found to be 3 % and 50 %respectively (Hansen *et al.*, 2007). In this study, ducks were the major source of phages which indicates that phage isolation is influenced more by the type of poultry tested (broiler or duck) than the sample type (intestine or abattoir sewage).

A study was performed to isolate phages from the skin and the caecal contents of chickens purchased from retail markets in different regions in Korea together with sewage and soils from several animal breeding grounds and abattoirs. *Campylobacter* phages were found in 20 % of chicken intestinal

samples but the environmental samples were negative when tested (Hwang *et al.*, 2009).

In New Zealand, retail chicken skin samples were tested for the presence of campylobacter phages and where found to be negative. In the same study, whole chicken rinses from 39 flocks were pooled, tested and 28.2 % were found to be phage positive (Tsuei *et al.*, 2007).

1.2.7 Survival of *Campylobacter* Bacteriophages

As with all other phages, *Campylobacter* phages can survive the same environmental conditions as their host, and they can survive with them from the chicken intestine to the surface of poultry meat (Atterbury *et al.*, 2003a). This means that anyone dealing with uncooked poultry meat is exposed to surviving phages. The property of extended survival of phages makes them useful therapeutic agents as they can be added to poultry drinking water or feed (Carvalho *et al.*, 2010b).

Phage capcids are made of proteins which are sensitive to acidity (Leverentz *et al.*, 2001), for them to survive the gastric barrier and resist the bile in the small intestine and colonize it. It was proposed that they should be combined with antacids prior to their use (Koo *et al.*, 2001) or by selection of those phages which can tolerate low pH. In a study performed by Loc Carrillo *et al.* (2005), *Campylobacter* phages were mixed with $CaCO_3$ before being applied orally to chickens. They persisted during the trial period and they were maintained with their host in the chicken intestine and their effectiveness was proven.

Unlike bacteria, phages are known to be resistant to detergents like SDS, sodium deoxycholate and Saponin, whereas chelating agents like citrate and EDTA accelerate the inactivation of phages at low salt concentration. Chemicals such as glycerine and ethanol when concentrated can cause rapid inactivation of phages (Adams, 1959).

D'Herelle (1926; cited in Adams, 1959) reported that phages can be inactivated when heated at 75 °C for 30 min. When the temperature was reduced to 70 °C, some survived. Thus defining the inactivation temperature will depend on the phage characteristics. As mentioned earlier, phages did not survive freezing in the study performed by Atterbury *et al.* (2003a). The inability to isolate phages from frozen chicken was suggested to be either because the skin surface structure was changed, which prevented phage attachment to it, or because phages lost their activity upon freezing.

1.2.8 Bacteriophage Therapy

Bacteriophage therapy is defined as the use of phages to treat bacterial infection. The development of phage therapy for controlling pathogenic bacteria was slow to develop in many countries because the use of antibiotics was well established. Another reason for the slower development of phage therapy was the lack of evidence of their efficiency (Sulakvelidze *et al.*, 2001), which is often due to the lack of understanding of phage biology and their replication dynamics as well as bacterial pathogenesis mechanisms and the nature of phage interaction with its host (Barrow *et al.*, 1998).

Currently phages are being developed for use as therapeutic agents against food pathogens. This is largely due to their wide distribution in the environment and simplicity in their isolation and propagation (Miller and Mandrell, 2005). Such therapy is attractive because it may overcome antibiotic resistance problems and reduces the use of chemicals in food production (Kutter and Sulakvelidze, 2005). For phages to be used in such therapy, a good understanding of the phages and their hosts is required (El-Shibiny *et al.*, 2005).

1.2.8.1 Advantages and Disadvantages of Bacteriophage Therapy

Phages have some properties which give them unique advantages over antibiotics. Such properties are that they are self-replicating and have self-limiting properties, where they replicate only when their hosts are present followed by their gradual removal from the environment when their hosts are not available (Connerton and Connerton, 2005). Other properties which make them of great value as therapeutic agents are their specificity and efficiency in killing their target bacteria without affecting the normal flora or their natural presence in the environment (Sulakvelidze *et al.*, 2001). Being highly specific, they have the ability to infect only specific species or strains of their hosts. Exception to this is found with *Listeria* phage A511 that is effective against the entire genus (Zink and Loessner, 1992). As phages are already present in the environment, their addition does not constitute the addition of any new biologically active entity thus reducing the possibility of the development of allergic response (Connerton and Connerton, 2006). In addition, they are

generally inexpensive to prepare (Connerton *et al.*, 2008) and no serious side effects have been described upon their use (Sulakvelidze *et al.*, 2001).

As with any other treatments, phage may have some negative impacts and problems such as development of phage resistance, which won't be a big problem because of the phages natural diversity and rapid replication. The availability of different phages will give technologists the choice to pick another phage that the strain will be sensitive to and as they replicate fast, selection for mutation can be done using the resistant strain which will result in a change in the host range to overcome many types of resistance (Connerton and Connerton, 2006). Another potential problem is the possibility of transferring pathogenic traits. This can be avoided by carefully selecting virulent phages (Connerton *et al.*, 2008). Lytic phages are good candidates for phage therapy as they infect the host and more phage particles will be produced and released upon cell lysis. On the other hand, lysogenic phages are not suitable for phage therapy as their DNA is integrated into the host DNA (Boyd and Brussow, 2002) and will have the ability to transfer genetic material from one bacterium to another. Some lysogenic phages may change the external membrane of bacteria that could result in an increase in their pathogenicity or resistance to such phages (Canchaya *et al.*, 2003). Phages themselves can adapt and continue to infect these pathogens (Sulakvelidze *et al.*, 2001). For example, a mutated phage tail fiber will allow binding to a mutant bacterial receptor or the mutated phage DNA can escape cleavage by mutant bacterial endonucleases (Carlton, 1999). Clearly, there is a balance that allows both host and predator to proliferate (Connerton *et al.*, 2004). Moreover, phages may

have too narrow a host range. For them to be used as a biocontrol agents, therefore their infectivity towards isolates that represent *Campylobacter* strains from broilers and humans should be considered (Hansen *et al.,* 2007). Screening bacteria against different phages to ensure that one of these phages will be lytic or the use of a combination of phages in a cocktail to lyse most or all bacterial types of a specific pathogen could overcome this problem. In addition, phages are usually prepared as crude lysates; thus, the presence of bacterial debris in such preparations together with endotoxins, even if present in low concentrations, could be injurious to patients where the therapy is to be used for human treatment. To solve this problem, density centrifugations and other methods of purifications (Carlton, 1999) including ion exchange chromatography should be performed. Lastly, phages in therapeutic preparations should be free from their hosts, thus oxidizing agents could be added to kill the host bacteria or sample heating could be done. Such applications can denature the protein coat and to ensure phage viability, the phage should be re-titrated over time. Other solutions to this problem involve the utilization of sterile filtration (Carlton, 1999; Sulakvelidze *et al.*, 2001).

1.2.8.2 Factors Affecting Bacteriophage Therapy

Phage replication depends both on their concentration (Levin and Bull 1996; Payne and Jansen, 2002; Bull *et al.*, 2002) and their host density. The required host density threshold is called 'bacteriophage proliferation threshold' (Payne and Jansen, 2002). It is important for all phages to proliferate sufficiently in order to cause a crash in the host bacterial population and reduce their numbers. Other factors affecting phage therapy include the inoculum size and

timing, phage adsorption rate and burst size (Levin and Bull, 1996; Weld *et al.*, 2004) which is the number of phages produced per cell which is usually in the order of 10-100 (Hadas *et al.*, 1997). Moreover, the phage adsorption kinetics to bacteria in the intestinal environment can be different from those determined experimentally on laboratory media, due to the viscosity of the mucus layer (Weld *et al.*, 2004).

1.2.8.3 Studies in Bacteriophage Therapy

In addition to the direct application of phages for treatment of bacterial infections in humans or animals, they may be used indirectly to prevent food-borne infection in animals used for food production, for example: their use in the reduction of pathogens including *Campylobacter*, *E. coli* or *Salmonella*, or they may be used during processing or directly applied to meat.

In veterinary medicine, phages have been used experimentally to control *Escherichia coli* infections in mice and calves. Smith and Hugins (1982) reported that phages that were isolated from sewage works and pig markets were successfully used in curing and reducing the number of deaths in mice infected with *E. coli* strains, and they were more effective than antibiotics tested in the same study such as ampicillin, chloroamphenicol and streptomycin. Moreover, the use of phages in protecting calves, piglets and lamb from diarrhoea caused by *E. coli* was reported in 1983. It was found that oral inoculation of phages one to eight hours after *E. coli* inoculation resulted in the protection of calves, piglets and lambs from diarrhoea and death and reduced the numbers of *E. coli* in the alimentary tract (Smith and Hugins,

1983). Barrow and others (1998) reported that phages were used in the protection of calves from septicaemia caused by *E. coli* as they delayed the bacterial appearance in the blood and lengthened their life span.

Phages were used in other studies in treating infections caused by bacterial strains other than *E. coli* as cited in Barrow *et al.* (1998), which includes: protection against *Salmonella typhi* infection in mice (Ward, 1943), the survival of mice infected by *Shigella dysenteriae* (Dubos *et al.*, 1943), wound treatments of burn patients caused by *Staphylococcus aureus* (Lowbury and Hood, 1953) and the control of *Pseudomonas aeruginosa* growth on pig skin (Soothill *et al.*, 1988).

Respiratory illness due to *E. coli* in chickens was shown to be reduced after treatment with phages (Huff *et al.*, 2003). Such phages were isolated from municipal waste treatment facilities and poultry processing plants and injected into chicken thighs having *E. coli* infection in single or multiple treatments, and were found to be effective in reducing the mortality rate and curing chickens when the disease was in its early stages, with multiple treatments being better than a single treatment. Phages were also used against meningitis in chickens caused by *E. coli*. Injecting infected birds with phages led to their fast multiplication on bacteria and prevented the massive multiplications of bacteria seen in the brains of untreated birds and as a result, the numbers of bacteria in the brains of treated birds declined; thus, the percentage of deaths was reduced (Barrow *et al.*, 1998).

Phages can be used to reduce the number of *Campylobacter* colonizing poultry. Reductions in *Campylobacter* numbers present in chicken caecal contents of between 2 $\log_{10}$ and 5 $\log_{10}$ (CFU g^{-1}) have been achieved (Loc Carrillo *et al.* 2005; Wagenaar *et al.,* 2005). Treatment of chicken just prior to slaughter could significantly decrease the number of campylobacters entering the human food chain. *Campylobacter* phage has been applied under experimental conditions to the surface of chicken skin for the same purpose (Goode *et al.*, 2003). Atterbury *et al.* (2003b) reported that under refrigerated conditions, campylobacters can be infected by phages but without being able to complete their replication cycle unless the conditions change to allow the host to increase its metabolic activities.

To find the effective dose of phages, a number of studies were performed using phages from group III (Loc Carrillo *et al.,* 2005; Scott *et al.,* 2007) in which it was found that *Campylobacter* colonization in broiler chickens can be reduced with the use of doses between 5 $\log_{10}$ and 9 $\log_{10}$ (PFU) with 7 $\log_{10}$ (PFU) to be the optimum effective dose. Rabinovitch *et al.* (2003) reported that phage aggregation and non-specific association with digesta or non- host bacteria could result in the highest doses being less effective. Their presence in higher concentrations will overwhelm the host in part of the intestine making them unavailable to continue further replication cycles in other parts of the gut as the phages need their host to multiply in order to be effective.

Group II and III phages have been used to reduce numbers of campylobacters in experimentally infected chickens. Both groups of phage succeeded in reducing *Campylobacter* numbers and have the advantage that they have

different host ranges (El-Shibiny *et al.*, 2009). Those from group II were used against *C. jejuni* and *C. coli* (El-Shibiny *et al.*, 2009; Carvalho *et al.*, 2010b) and those from group III (Loc Carrillo *et al.*, 2005; Scott *et al.*, 2007) were used in therapy against *C. jejuni* only.

Phages were used experimentally by Wagenaar and colleagues (2005) with broiler chickens, to examine the use of phages as preventive and therapeutic agents. In this work, chickens were infected with *C. jejuni* after four days of phage infection in the preventive group. On the other hand, in the therapeutic group, they were infected with phages five days after *C. jejuni* infection. It was found that phages do not prevent colonization. Instead, they delayed it in the preventative groups. Therapeutically, phages reduced the colonization levels by several orders of magnitude, thus they have the potential to be used for the control of campylobacteriosis arising from poultry contamination.

On plant crops, phages have been used to reduce *Salmonella* contamination when experimentally applied to fresh cut apples and melons at 5, 10 and 20 °C. It was found that the reduction of *Salmonella* contamination by phages was temperature dependent and the greatest *Salmonella* reduction, on melon, was achieved at temperatures of 5 and 10 °C. The titre of the phage remained stable throughout the experiment. In contrast, no reduction in *Salmonella* numbers or recovery of phages was observed in the case of apples, which was probably due to their natural acidity (Leverentz, *et al.*, 2001).

1.2.8.4 Commercially Available Bacteriophages

Phages have been used as therapeutic, prophylactic and disinfectant agents (Alisky *et al.,* 1998). A few commercial products are available from various biotechnology companies. Agriphage, a product supplied by Omnilytics company is used to target the bacterial spots on crops as tomatoes and pepper specifically for *Xanthomonas campestris* pv. Vesicatoria or *Pseudomonas syringae* pv. tomato strains. In the food sector, many products are available against different bacterial strains including: BioTector which is supplied by Cheiljedang Corporation to be applied to animal feed against *Salmonella* in chicken. Moreover, Intralytix Company produces Ecoshield that targets *E. coli* in food and ListShield against *Listeria monocytogenes* which can also be targeted by LISTEX P100 which is produced by EBI food safety company (Monk *et al.,* 2010).

1.2.9 Disruption of Biofilms by Bacteriophages

Bacterial strains of one or several species live as biofilms attached to a surface and embedded in an extra-cellular polymeric matrix in their normal habitats, which gives them protection against a number of stresses in their environment including: desiccation, heating, disinfectants and acidic conditions, increasing their chances to survive. Phages have been used to reduce the biofilms of different bacterial strains (Hibma *et al.,* 1997; Hughes *et al.,* 1998) including *Campylobacter* biofilms (Siringan *et al.,* 2011). In addition to that, engineered phage enzymes have been used to disperse biofilms by breaking down extra-cellular polymeric matrix components (Lu and Collins, 2007). Therefore,

phages are promising agents in controlling *Campylobacter* biofilms and they can be used for food safety applications in industry (Connerton *et al.*, 2011).

1.3 Bacteriophage Lytic Enzymes (Lysins)

1.3.1 Introduction

Lysins are defined as enzymes produced by phages to break down the bacterial cell wall (Jado *et al.*, 2003; Horgan *et al.*, 2009) in order to release phage progeny (Fischetti, 2008) with the aid of intermediate protein molecules called holins (Young, 1992; Sable and Lortal, 1995; Loessner *et al.*, 1997).

Lysins have been studied in Gram-positive bacteria, for example: *B. subtilis* (Ward *et al.*, 1982; Garvey *et al.*, 1986) and *S. pneumoniae* (Garcia *et al.*, 1987; Romero *et al.*, 1990) and in Gram-negative bacteria for example: *Pseudomonas aeruginosa* PAO1 (wild type), *Pseudomonas aeruginosa* Br667 (multidrug resistant burn wound isolate), *E. coli* XL1-Blue MRF and *Salmonella enteric* Serovar Typhimurium LT2 (Walmagh *et al.*, 2012). The main differences between the different types of lysins studied are their specificity and whether they attack the thick Gram-positive cell wall or the thin peptidoglycan layer of the Gram-negative bacteria, which is protected by an outer membrane (Schuch *et al.*, 2009). Lysins are known to be more effective at killing Gram-positive bacteria because of lack of the outer membrane and the direct contact with the cell wall and the peptidoglycan (Fischetti, 2006), whereas in case of Gram-negative bacteria the outer membrane prevents this interaction (Fischetti, 2008).

1.3.2 Lysin Structure

Lysins are categorized by their domain structure (Fischetti, 2006). Most of the enzymes are composed of two domains; N-terminal and C-terminal domains separated by a short linker (Fischetti, 2008).

The N-terminal domain possesses the catalytic activity of the enzyme which breaks down one of the main bonds in the peptidoglycan in the cell wall (Young, 1992); which include lysozymes (N-acetylmuramidases), glycosidases (N-acetyl-B-D-glucosaminidases) which hydrolyse the β-1-4-glucosidic bond in the sugar moiety, endopeptidases (L-alanoyl-D-glutamate) that break down the peptide cross-bridge and amidases (N-acetyl-muramoyl-L-alanine amidase) that break the amide bond between the sugar and peptide moieties (Fenton *et al.,* 2010). Lysins that are specific for Gram-negative strains are composed of a single 15-200 kDa catalytic domain, whereas those targeting Gram-positive strains are 25-100 kDa in size and may have one or more catalytic domains (Schuch *et al.,* 2009). Reported lysins having two catalytic domains from *Staphylococcus aureus* are; LysK, Phill and MV-L (Navarre *et al.*, 1999; O'Flaherty *et al.,* 2005; Rashel *et al.*, 2007). The C-terminal cell binding domain confers substrate specificity (Fischetti, 2006). It is responsible for the enzyme attachment to its carbohydrate substrate in the bacterial cell wall through non-covalent binding, and it is needed for the lytic activity of some lysins, but this is not always the case (Fenton *et al.,* 2010). Some lysins such as Lys K produce higher lytic activities when their binding domains are removed. Horgan *et al.,* (2009) reported that the C-terminal binding domain may be

responsible for limiting the activity of the N-terminal catalytic domain which allows it to function only when it is bound to its target in the cell wall.

Garcia *et al.* (1990) suggested that lysins with different bacterial and catalytic specificities can result when different enzyme domains swap. Examples of this can be seen for the *Streptococcus pneumoniae* phages. The catalytic domains of their lytic enzymes could be swapped resulting in a new enzyme. This enzyme has the same binding domain for *S. pneumoniae* but with the ability to cleave different peptidoglycan bonds.

1.3.3 Mechanism of Action

The phage lytic system consists of one or more peptidoglycan hydrolases or lysins that act on the cell wall and holin protein molecule, both of which are essential for the cell lysis. During the late stage of the lytic cycle, lysins accumulate in the cytosol and hydrolyse the peptidyglycan in the cell wall resulting in the release of mature phage progeny (Fenton *et al.,* 2010). As they do not have signal sequences, lysins are not translocated across the cytoplasmic membrane. Therefore they need a second phage gene product in the lytic system to reach their targets within the peptidoglycan layer. This product is the holin (Wang *et al.*, 2000) that acts by forming holes in the membrane to allow the passage of endolysins to the cell wall (Payne and Hatfull, 2012). Also, it can control the timing of lysis (Wang *et al.,* 2000). Once the lysin reaches the cell wall, it breaks down the peptidoglycan layer. The osmotic pressure differences between the cells and the surrounding environment cause the cytoplasmic membrane to rupture through hypotonic lysis (Fischetti, 2006).

For large DNA phages, during phage production, the lysins accumulate in the cytoplasm. The cell membrane will be disrupted when holin molecules are expressed and inserted into the cytoplasmic membrane. At this stage the lysins from the cytoplasm will reach the peptidoglycan layer (Young *et al.,* 2000; Bernhardt *et al.,* 2001) and break covalent bonds in the cell wall that are responsible for maintaining its structure. This leads to bacterial lysis and progeny phage release (Schuch *et al.,* 2009). In contrast, in phages with limited coding capacity, the phage-encoded proteins will interfere with the bacterial enzymes that are responsible for peptidoglycan synthesis (Young *et al.*, 2000; Bernhardt *et al.*, 2001) thus leading to a misassembled cell wall and cell lysis.

1.3.4 Lysin Specificity

Lysins are highly specific and will only kill the species (or subspecies) of the bacteria from which the phage that encoded them were produced (Loeffler *et al.,* 2001) without affecting normal flora. For them to function, the binding domain is required to bind to its target within the cell wall, thus offering some degree of specificity, as the target will only be found in enzyme-sensitive bacteria (Fischetti, 2008). Once the binding is established, the catalytic domain can break down the cell wall (Loeffler *et al.,* 2001).

In some cases, lysins can have a broad lytic activity, as in the enterococcal phage lysins. It was reported that they have the ability to kill enterococci as well as some other Gram-positive bacteria including *Streptococcus pyogenes*, group B streptococci and *Staphylococcus aureus*. Such ability makes this group

one of the broadest lysins identified, even though the activity on *Enterococci* is higher than on the other genera (Yoong *et al.*, 2004).

1.3.5 Consideration of Lysin-Based Therapies

For lysins to be used therapeutically, their properties, including toxicity, immuonogenicity, resistance and synergy, should be considered. Lysins are not known to be toxic against humans or animals. This was demonstrated using lysin treatment in a systemic infection mouse model where no signs of toxicity were evident (Nelson *et al.*, 2001; Jado *et al.*, 2003). Similarly, in pre-clinical trials *in vivo*, there were no harmful, abnormal or irritant side effects shown (Loessner, 2005). The use of lysins stimulates the immune response which leads to reduction in their activities. This was demonstrated when lysin Cpl-1 from *Streptococcus pneumoniae* was used against rabbit hyper immune serum. Its activity was reduced because of immune system stimulation (Loeffler *et al.*, 2003). Bacterial resistance to lysins is not expected as phages have evolved alongside their hosts and these proteins are absolutely needed for the release of the progeny from the host cell and they target peptidoglycan which is essential for bacterial viability (Fenton *et al.*, 2010).

The performance of lysins can be improved when used in combination with other lysins *in vitro* and *in vivo*. The use of pneumococcal Cpl-1 (lysozyme) and Pal (amidase) in synergy in a murine sepsis model (Jado *et al.*, 2003), and the use of *Staphylococcus* lysin LysK and lysostaphin together (Becker *et al.*, 2008) resulted in greater lysis than using them separately (Jado *et al.*, 2003). Similarly, lysins can perform better in synergy with antibiotics and this was

demonstrated in a number of studies. The activity of Pneumococcal enzymes as antimicrobial agent was improved when combined with antibiotics. For example CPl-1 with penicillin and gentamicin (Djurkovic *et al.*, 2005) and LytA with cefotaxime and moxifloxacin (Rodriguez-Cerrato *et al.*, 2007).

1.3.6 Potential Applications of Lysins

These enzymes have many potential applications including the control of pathogenic bacteria on human mucous membranes (Fischetti, 2006). Examples of pathogens that can be treated with lysins include; *Streptococcus pneumoniae* (Loeffler *et al.*, 2001), *S. pyogenes* (Nelson *et al.*, 2001), *Bacillus anthracis* (Schuch *et al.*, 2002), *Staphylococcus aureus* (Horgan *et al.*, 2009) and *Clostridium difficile* (Mayer *et al.*, 2008). In addition, such lysins could be used in the food industry (Fischetti, 2006) against pathogens causing food poisoning which will lead to economic loss in poultry production. *Clostridium perfringens* in poultry intestines was controlled by Ply3626 lysin (Zimmer *et al.*, 2002). In veterinary medicine, lysins were used against bovine mastitis which causes death in dairy cows and contamination of their milk that can be used in food production. Ply700 showed lytic activity against mastitis caused by *Streptococci* in raw and pasteurized milk respectively (Celia *et al.*, 2007). Plant protection against phytopathogens was achieved using Coliphage T4 lysins against *Erwinia carotovora* that cause soft rot in potatoes (Borysowski *et al.*, 2006). Lastly, with the increase of antibiotic resistance, lysins can be used as alternative antimicrobial agents against infectious diseases (Fenton *et al.*, 2010). It was reported that Gram positive bacteria repeatedly exposed to low concentrations of lysins did not result in the recovery of resistant strains even

after 40 cycles *in vitro* (Fischetti, 2008) regardless of whether they were grown in liquids or on agar plates (Nelson *et al.*, 2001; Schuch, 2009) which is a distinct advantage over the use of antibiotics.

Lysins are currently considered to have great future potential in controlling bacterial pathogens, but it is important to remember that there will always be a need to find new ways both to prevent and treat pathogen infections (Fischetti, 2008).

Objectives

- Isolation and characterization of *Campylobacter* phages and their hosts from chicken faeces.
- Identification of possible lysin genes from CP220 phage that was isolated from poultry excreta, followed by their amplification, cloning and sub-cloning in expression and non-expression vectors.
- Molecular characterization of *Campylobacter* phages including; CPX, CP8, CP20, CP30 and CP34 through DNA amplification and sequencing of selected genes taking the availability of CP220 and CPt10 phages sequences that were published recently by Timms *et al.* (2010) as an advantage.
- Complete genome Sequencing of CPX phage that was a originally a co-isolate of phage CP220 before separation, independent propagation and purification using caesium chloride density gradient centrifugation and to enable its comparison to CP220 phage and other phages available in the data bases.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Culture Media Preparation

All types of media used were prepared in reverse osmosis water (RO water) followed by autoclaving at 121 °C, 15 psi for 20 min using the liquid cycle. The autoclaved media were cooled to 50 °C before the addition of any supplement if required. After cooling, the media were kept at 4 °C until needed.

2.1.1 Blood Agar (BA)

Blood Agar base No. 2 (Oxoid, Basingstoke, UK) was prepared according to the manufacturer's instructions. After sterilization and cooling to 50 °C, 5 % (V/V) defibrinated horse blood (TCS Biosciences LTD, Botolph Claydon, UK) was added, then the media was mixed thoroughly and poured into Petri dishes.

2.1.2 Charcol Cefoperazone Deoxycholate Agar (CCDA)

Campylobacter blood-free selective agar (Oxoid) was prepared according to the manufacturer's instructions, sterilized and supplemented with cefoperazone (ProLabs Diagnostics, Wirral, UK), which was dissolved in 5 ml of sterile water, mixed thoroughly and poured into Petri dishes.

2.1.3 New Zealand Casamino Acids Yeast Medium (NZCYM) Basal Agar

NZCYM broth (Difco, Oxford, UK) was prepared and supplemented to a final concentration 1.2 % (W/V) with bacteriological agar No. 1 (Oxoid) according to the manufacturer's instruction, sterilized, mixed thoroughly and poured into Petri dishes.

2.1.4 NZCYM Overlay Agar

NZCYM broth (Difco) was prepared according to the manufacturer's instruction and supplemented with bacteriological agar No. 1 (Oxoid) to a final concentration of 0.6 % (W/V), sterilized, mixed thoroughly and stored in 200 ml amounts until required. It was then melted and dispensed into 5 ml amounts and cooled to 50 °C and used.

2.1.5 Luria-Bertani (LB) Agar

LB (Lennox) broth (Difco), supplemented with bacteriological agar No. 1 to a final concentration of 1.5 % w/v, was prepared according to the manufacturer's instructions and sterilized. Selective antibiotics; were added prior pouring in Petri dishes.

2.1.6 Luria-Bertani (LB) Broth

LB (Lennox) broth (Difco) was prepared according to the manufacturer's instructions. Ten millilitre aliquots were dispensed in 30 ml universal bottles and sterilized. Selective antibiotics were added before their use.

2.1.7 Nutrient Broth (NB)

Nutrient broth No. 2 (Oxoid) was prepared according to the manufacturer's instructions, sterilized and mixed thoroughly.

2.2 Storage Media Preparation

2.2.1 *Campylobacter* Storage Medium

Nutrient broth (NB) No. 2 (Oxoid) was prepared according to the manufacturer's instruction, sterilized and supplemented with glycerol (Fisher, Loughbrough, UK) to give a final concentration of 15 % glycerol (V/V), dispensed in 1 ml cryovials and stored at room temperature (20 °C) before use.

2.2.2 Bacteriophage Storage Medium

NZCYM broth (Difco) was prepared according to the manufacturer's instruction, sterilized and supplemented with glycerol (Fisher) to give a final concentration of 5 % glycerol (V/V), dispensed in 1 ml cryovials and stored at room temperature (20 °C) before use.

2.2.3 Clone Storage Medium

A 10 ml LB broth (Difco) was prepared according to the manufacturer's instruction, sterilized and supplemented with glycerol (Fisher) to give a final concentration of 50 % glycerol (V/V), 250 µl aliquots were dispensed in 1 ml cryovials and stored at room temperature (20 °C) before use.

2.3 Solutions and Buffers

2.3.1 Salt Magnesium (SM) Buffer

SM buffer (0.1 M NaCl, 8 mM $MgSO_4.7\ H_2O$, 0.002 % gelatin (w/v), 50 mM Tris-Cl) was prepared by mixing 5.8 g NaCl (Fisher), 2 g $MgSO_4.7\ H_2O$ (Fisher), 5 ml of 2 % (w/v) gelatin (Sigma-Aldrich, Poole, UK) and 50 ml of

1.0 M Tris-HCl (pH 7.5; Sigma-Aldrich). The volume was made up to 1 l with RO water after which it was sterilized and stored at room temperature (20 °C).

2.3.2 Magnesium Sulphate ($MgSO_4$)

A 1 M solution of $MgSO_4$ (Sigma-Aldrich) was prepared by dissolving 12 g $MgSO_4$ in 100 ml RO water, sterilized and stored at room temperature (20 °C).

2.3.3 Tris Acetate EDTA Buffer (TAE) pH 8.0

A 10 X TAE buffer stock solution was prepared by dissolving 48.4 g of Trizma base (Sigma-Aldrich); 10 ml of glacial acetic acid (Sigma-Aldrich) and 2.92 g of EDTA (Sigma-Aldrich) in 750 ml of RO water, the pH was adjusted to 8.0 and the volume was adjusted to 1 l with RO water. After which it was sterilized and diluted 1:10 in RO water when required.

2.3.4 Tris EDTA Buffer (TE) pH 7.5

A 10 X TE buffer stock solution (100 mM Tris-HCl, 10 mM EDTA) was prepared by dissolving 12.1 g of Trizma base (Sigma-Aldrich) and 3.7 g of EDTA (Sigma-Aldrich) in 750 ml of RO water, the pH was adjusted to 7.5 and the volume was adjusted to 1 l with RO water. Then, it was sterilized and diluted 1:10 in RO water when required.

2.3.5 Tris Borate EDTA Buffer (TBE) pH 8.0

A 5 X TBE buffer stock solution (445 mM Tris-base, 445 mM boric acid, 10 mM EDTA) was prepared by dissolving 54.0 g of Trizma base (Sigma-Aldrich); 27.5 g of boric acid and 2.93 g of EDTA (Sigma-Aldrich) in 750 ml

RO water, then the pH was adjusted to 8.0 and the volume was adjusted to 1 l of RO water. This solution was sterilized and diluted 1:10 in RO water when required.

2.3.6 Phosphate Buffered Saline (PBS)

Phosphate buffered saline (Oxoid) containing: sodium choloride 0.16 M; potassium chloride; 0.003 M; disodium hydrogen phosphate 0.008 M; potassium dihydrogen phosphate 0.001 M, was prepared by dissolving 1 tablet per 100 ml RO water according to manufacturer's instructions and sterilized. PBS was stored at room temperature (20 °C) until required.

2.3.7 Ethylenediaminetetraacetic Acid (EDTA) pH 8.0

A 500 mM stock solution was prepared by dissolving 186.12 g EDTA (Sigma-Aldrich) in 750 ml RO water, the pH was adjusted to 8.0 and the volume was adjusted to 1 l with RO water. The solution was then sterilized and stored at room temperature (20 °C) until required.

2.3.8 Isopropyl-B-D-Thiogalactoside (IPTG)

A 100 mM IPTG was prepared by dissolving 0.12 g IPTG (Roche, Welwyn Garden City, UK) in 5 ml RO water, sterilized through a 0.2 µm membrane filters (Sartorius, Epsom, UK) and stored at 4 °C until required.

2.3.9 Lysozyme

Lysozyme from chicken egg white (Sigma-Aldrich) was used to prepare a solution at 50 mg ml^{-1}, sterilized through 0.2 µm membrane filters (Sartorius), dispensed in 500 µl portions and stored at -20 °C until required.

2.3.10 Calcium Chloride ($CaCl_2$) Solution

This solution was prepared by dissolving 60 ml of 1 M $CaCl_2$ (Fisher), 3.0237 g piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES; Sigma-Aldrich) and 150 ml 100 % glycerol (Fisher) in 750 ml RO water. The pH was adjusted to 7.0 and the volume was adjusted to 1 l to give final concentrations of 60 mM $CaCl_2$; 100 mM PIPES; 15% glycerol, sterilized and stored at 4 °C until required.

2.3.11 Ammonium Acetate Solution

Stock solutions of 7.5 M and 100 mM ammonium acetate (Sigma-Aldrich) were prepared, sterilized and stored at room temperature (20 °C) until required.

2.3.12 Cells Lysis Solution

Lysis solution (20 % w/v sucrose, 30 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mg ml^{-1} lysozyme) was prepared by dissolving 20 g sucrose (Sigma-Aldrich; 20 % W/V); 6 ml of 0.5 M Tris-HCl (Sigma-Aldrich) pH 8.0 (30 mM); 200 µl of 0.5 mM EDTA (2.3.7; 1 mM) in up to 100 ml RO water, sterilized and stored at 4 °C. Prior to its use, lysozyme (2.3.9) was added to a concentration of 1 mg ml^{-1}.

2.3.13 Tris-HCl

Stock solutions of 1 M, 0.5 M or 100 mM Tris(hydroxymethyl)aminomethane (Sigma-Aldrich) were prepared, adjusted to the pH required with concentrated HCl (Fisher), made up to the correct volume then sterilized and stored at room temperature (20 °C).

2.3.14 Triton-X-100

A 1 % Triton-X-100 (Sigma-Aldrich) solution was prepared with RO Water, sterilized and stored at room temperature (20 °C) until required.

2.3.15 Tricine Gels Stain Solution

Stain solution was prepared by dissolving 2.5 g Coomassie Brilliant Blue (0.25 %; Sigma-Aldrich) in 10 % glacial acetic acid (Fisher), 45 % methanol (Fisher). The volume was adjusted to 1 l RO water. The solution was stored at room temperature (20 °C) until required.

2.3.16 Tricine Gels Destain Solution

A destain solution was prepared by mixing 10 % glacial acetic acid (Fisher), 45 % methanol (Fisher) and adjusted to 1 l with RO water. The solution was stored at room temperature (20 °C) until required.

2.3.17 Ethidium Bromide

A Stock solution of ethidium bromide (100 mg ml^{-1}; Sigma-Aldrich) was prepared in RO water, and stored at 4 °C. When required, it was used at 1 μg ml^{-1} final concentration.

2.4 Antibiotics

2.4.1 Ampicillin

A stock solution of ampicillin (100 mg ml^{-1}; Sigma-Aldrich) was prepared in RO water, sterilized through a 0.2 μm membrane filters (Sartorius) and stored at 4 °C until required. When required, it was used at 100 μg ml^{-1} final concentration.

2.4.2 Chloramphenicol

A stock solution of chloramphenicol (30 mg ml^{-1}; Sigma-Aldrich) was prepared in 100 % ethanol (Fisher), sterilized through a 0.2 μm membrane filters (Sartorius) and stored at -20 °C until required. When required, it was used at 30 μg ml^{-1} final concentration.

2.5 Growth of Campylobacters

2.5.1 Atmosphere Generation

Microaerobic atmospheres were generated using either the evacuation replacement method where the jars are evacuated to -20 Hg and replaced with anaerobic gas mixture consisting of 85 % nitrogen, 10 % carbon dioxide and 5 % hydrogen to produce an atmosphere containing 83 % v/v nitrogen, 7 % v/v

carbon dioxide, 7 % oxygen and 3 % v/v hydrogen, or through incubation in the Modular Atmospheric Controlled System (MACS; Don Whitley Scientific, Shiply, UK) which was supplied with a gas mixture of 85 % v/v nitrogen, 10 % v/v carbon dioxide and 5 % oxygen.

2.5.2 Growth and Sub-Culture of Campylobacters

Campylobacters were routinely sub-cultured on blood agar (2.1.1) and incubated at 42 °C under microaerobic conditions (2.5.1). The blood agar plate cultures were stored for up to 28 d at 4 °C also under microaerobic conditions.

2.5.3 Gram Stain

A smear was prepared by mixing an isolated colony with drop of water on glass slide, where it was dried and fixed with heat. The slide was immersed in crystal violet solution (Prolabs Diagnostics, Gram stain kit) for 1 min, followed by Lugol's iodine (Prolabs Diagnostics, Gram stain kit) for 30 s and finally decolorized with methylated spirit (Fisher) for 1 min and counter stained in carbol fuchsin (Prolabs Diagnostics, Gram stain kit) for 1 min. In between each step, excess solution was removed by rinsing with water. Slides were examined using light microscopy.

2.5.4 Isolation of *Campylobacter* from Chicken Excreta

Samples taken from the center of the chicken faeces, were directly plated onto CCDA plates (2.1.2). All plates were incubated at 42 °C under microarobic conditions (2.5.1) for 48 h. Colonies were Gram-stained (2.5.3) to check for the presence of *Campylobacter*. Colonies from plates showing positive growth

were sub-cultured on to BA (2.1.1) plates and incubated at 42 °C under microaerobic conditions (2.5.1) for 48 h. Once pure colonies were obtained, they were collected and frozen at -80 °C in *Campylobacter* storage medium (2.2.1).

2.6 *Campylobacter* Bacteriophage

2.6.1 Bacterial Lawn Preparation

Lawns were prepared using a modified method from Frost *et al.* (1999). Different strains were grown as described in section 2.5.2 on BA (2.1.1) at 42 °C for 24 h under microaerobic conditions (2.5.1). Cells were collected using a sterile swab into 10 ml of sterile 10 mM $MgSO_4$ freshly prepared from stock (2.3.2). Portions of the bacterial suspension (500 µl) which contained approximately 10^{10} colony forming units (CFU) per ml were transferred into 5 ml NZCYM overlay agar (2.1.4) which had been melted and kept at 50 °C until used. The inoculated agar was immediately poured on pre-dried NZCYM base agar plates (2.1.3). The plates were rotated gently to produce an even lawn and allowed to set at room temperature (20 °C). After which, they were dried at 42 °C for 20 min before use.

2.6.2 Isolation of *Campylobacter* Bacteriophage

This method was modified from Salama *et al.* (1989). Faeces samples (0.5 g) were suspended in 5 ml SM buffer (2.3.1) and allowed to stabilize at 4 °C with gentle shaking, overnight. A 1 ml portion was transferred to an Eppendorf tube and centrifuged at 13,000 *g* for 10 min, followed by filtering the supernatant using 0.2 µm pore size membrane filter (Sartorius). A 10 µl aliquot was

dispensed onto a previously prepared bacterial lawn containing the universal phage propagation strain *C. jejuni* PT14 (2.6.1), and incubated 42 °C for 24 h under microaerobic conditions (2.5.1). Plates were examined for plaque formation. Plaques were collected using a 1 ml Gilson tip and dispersed in 500 µl SM buffer (2.3.1) which was then used for phage propagation.

2.6.3 Propagation and Concentration of *Campylobacter* Bacteriophage

Campylobacter phages were propagated using the modified method from Frost *et al.* (1999). Bacterial lawns were prepared as previously described (2.8.1).

An aliquot of phage suspension was mixed with 400 µl of bacterial suspension (2.6.1) and incubated at 42 °C for 20 min. The mixture was added to 5 ml NZCYM molten overlay agar (2.1.4) and poured onto NZCYM base agar (2.3.1) and allowed to set at room temperature (20 °C) for 20 min. Plates were inverted and incubated at 42 °C for 24 h under microaerobic conditions (2.5.1). After plaque formation, the phages were collected by the addition of 5 ml SM buffer (2.3.1) to the surface of each plate and the phage allowed to elute into the buffer by shaking the plates at 4 °C overnight. The phage suspension was collected, filtered using 0.2 µm membrane filters (Sartorius) and concentrated as described by Sambrook *et al.* (1989) where the phage suspension was centrifuged at 40,000 *g* for 2 h at 4 °C using a Beckman JA20 rotor using a Beckman J2-21 centrifuge (Beckman, High Wycombe, UK). Pellets were dispersed in (500 µl) SM buffer (2.3.1) and phage allowed to elute in the buffer at 4 °C overnight with gentle shaking.

2.6.4 Bacteriophage Titration

Ten-fold serial dilutions of the propagated phages were prepared in SM buffer (up to 10^{-8}). A 10 µl aliquot of each dilution was dispensed onto the prepared lawns (2.6.1) using the Miles and Misra (1938) technique. The liquid was allowed to be absorbed and plates were incubated at 42 °C for 24 h under microaerobic conditions (2.5.1). The number of plaque forming units (PFU) per ml in the phage suspension was calculated.

2.6.5 Bacteriophage Clean-up Using Ammonium Acetate for Electron Microscopy

The propagated phage (2.6.3) was treated with ammonium acetate (2.3.9) as described by Ackermann (2005) prior to examination by electron microscopy. A 40 ml phage sample in SM buffer was centrifuged using a JA20 rotor in a J2-21 Beckman centrifuge at 30,000 *g*, 4 °C, 2 h without the brake. Then the supernatant was discarded carefully and without disturbing the pellet, 20 ml of 0.1 M ammonium acetate (2.3.9) was added and the solution was centrifuged at 25,000 *g*, 4 °C, 1 h without the brake. This step was repeated after which the pellet was dispersed in 1 ml SM buffer (2.3.1) and kept on a shaker at 4 °C overnight.

2.6.6 Pulsed Field Gel Electrophoresis (PFGE) to Determine Bacteriophage Genome Size

The method used to prepare DNA for separation by PFGE was modified from the standard protocols of Sambrook *et al.* (1989). Phage suspension (2.6.3; 50 µl) was mixed with 10 µl of 20 mg ml^{-1} proteinase K (Sigma-Aldrich)

solution and 50 µl of 1.2 % molten pulsed field certified ultra-pure agarose (Bio-Rad, Hemel Hempstead, UK) in TE buffer (2.3.4), mixed well and dispensed into moulds and allowed to solidify. Once solidified, blocks were removed and placed in Eppendorf tubes containing 1 ml lysis solution [100 µl proteinase K of 20 mg ml^{-1} (Sigma-Aldrich) in 50 mM EDTA pH 8.0 (2.3.7), 50 mM Tris-HCl (pH 8.0) (Sigma-Aldrich), 1 % sarcosyl (Sigma-Aldrich)] and incubated at 55 °C for 1 h. The lysis solution was discarded and 1 ml water was added. The solution was incubated at 55 °C for 15 min, then blocks were washed 3 times with washing buffer (20 mM Tris-HCl [pH 8.0] ;Sigma-Aldrich), 50 mM EDTA pH 8.0 (2.3.7) and incubated at 55 °C for 15 min between each wash.

Each block was cut into 3 mm slices, transferred to the wells of a 1 % agarose (Bio-Rad) gel prepared in 0.5 X TBE (2.3.5) together with a block of 50-1000 kb pulse marker (Sigma). The wells were sealed with molten agarose and allowed to set, after which they were transferred to the electrophoresis apparatus and run in 0.5 X TBE using the following program: 10-30 s over 17 h using 6.0 v/cm with buffer circulating at 14 °C in a BioRad CHEF-DR® II system. At the end of the run, the gel was stained with ethidium bromide (1 µg ml^{-1}) solution (2.3.17) for 30 min and the image was recorded using a ChemiDoc XRS Imager using the Quantity One program (Bio-Rad).

2.6.7 Bacteriophage DNA Preparation

To 200 µl of phage (2.6.3) sample containing 10^8 PFU ml^{-1}, 20 µl proteinase K (10 mg ml^{-1}; Sigma-Aldrich) and 200 µl detergent solution consisting of 2 % sarcosyl (Sigma-Aldrich) , 0.4 % SDS (Sigma-Aldrich), 20 mM Tris-HCL (pH 8.0; Sigma-Aldrich) and 200 mM EDTA (pH 8.0; Sigma-Aldrich) were added and incubated at 55 °C for 2 h after which 500 µl phenol (Sigma-Aldrich) was added, mixed by inversion and centrifuged at 13,000 *g* for 1 min using a Biofuge Pico bench-top centrifuge (Kendro Laboratory Products, Bishops Stortford, UK). The supernatant was transferred to a clean eppendorf tube and mixed with 500 µl chloroform (Sigma-Aldrich), centrifuged under the same conditions. This step was repeated. Ammonium acetate (7.5 M) was added in amount equal to 1/10 of sample volume, and twice of the volume of 100 % cold ethanol was also added, mixed gently and kept at -20 °C overnight.

The sample was centrifuged at 13,000 *g* for 10 min. The supernatant was discarded and the pellet was washed with 70 % ethanol followed by centrifugation at 13,000 *g* for 10 min. The supernatant was discarded again and the pellet was dried at 37 °C for 15 min after which the pellet was dispersed in 50 µl 1 X TE (2.3.4) buffer. The concentration of prepared DNA was measured using an ND-1000 spectrophotometer 'Nanodrop' (Labtech, Ringmer, UK) at λ 260 and stored at 4 °C until needed.

2.6.8 Whole Genome Amplification Using REPLI-g Kit

The prepared DNA (2.6.8) was pre-amplified using REPLI-g mini prep kit (Qiagen, Crawley, UK) following their instructions. Briefly, to 5 µl of DNA 5 µl buffer D_1 (9 µl buffer DLB and 32 µl nuclease free water) was added, mixed by vortexing, centrifuged briefly and incubated at room temperature (20 °C) for 3 min. To this, a 10 µl buffer N_1 (12 µl stop solution and 68 µl nuclease free water) was added, mixed by vortexing and centrifuged briefly. Then 30 µl master mix (29 µl REPLI-g mini reaction buffer and 1 µl REPLI-g mini DNA polymerase) was added and incubated at 30 °C for 10-16 h. The DNA polymerase was deactivated at 65 °C for 3 min and the samples were stored at -20 °C until required.

2.6.9 Polymerase Chain Reaction (PCR)

Phage DNA was prepared (2.6.7) and amplified. The reaction mixture was prepared as follows:

Reagent	Stock Concentration	Final Concentration	Volume Used
PCR buffer (AB gene, Surry, UK)	10 X	1 X	5 µl
$MgCl_2$ (AB gene)	25 mM	2.25 mM	4.5 µl
dNTPS (Promega, Southampton, UK)	25 mM each	2.5 mM each	5 µl
DNA	50 ng μl^{-1}	1 ng μl^{-1}	1 µl
Forward Primer (Eurofins MWG)	100 pmol μl^{-1}	2 pmol μl^{-1}	1 µl
Reverse Primer (Eurofins MWG)	100 pmol μl^{-1}	2 pmol μl^{-1}	1 µl
Taq Polymerase (Fisher)	1 U μl^{-1}	0.02 U μl^{-1}	1 µl
Sterile Distilled Water	-	-	31.5 µl

At the end of the run, the products were stored at -20 °C for further analysis. The primers (Eurofins MWG, Ebersberg, Germany) were designed and used for amplification of each gene sequence.

2.6.10 Agarose Gel Electrophoresis

Agarose (Biogene, Cambridge, UK) was dissolved in TAE buffer (2.3.3) and heated to prepare gels of different concentrations (0.8, 1 and 1.5 %). The mixture was cooled to approximately 50 °C and ethidium bromide to a final concentration of 1 $\mu g\ ml^{-1}$ was added. This was followed by pouring the gel into a minigel cast (Anachem, Luton, UK) with 1.5 mm well combs and allowed to set. The well combs were removed and the gel transferred to an electrophoresis chamber filled with TAE buffer (2.3.3).

The PCR (2.6.10) products (20 %) were mixed with 6 X loading dye (Promega) and loaded in the gel wells together with DNA markers (Promega) and run at 85 V (70 V for low melting gels) for 30-45 min based on the migration rate.

2.6.11 PCR Product Purification using Gel Extraction Kit

PCR products (2.6.10) were run on a 0.8 % low melting point agarose (Melford Laboratories Ltd, Ipswich, UK) gel containing $1\mu g\ ml^{-1}$ ethidium bromide (2.3.17) in 1 X TAE (2.3.3) followed by its purification if required using QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Briefly, the regions of gel containing the product were excised using a clean, sharp scalpel and any extra agarose removed. To 1 volume of the

gel, 3 volumes of QG buffer were added and incubated at 50 °C for 10 min, after which 1 volume of isopropanol (Sigma-Aldrich) was added to the sample and mixed. The sample was applied to a QIAquick column and centrifuged at 13,000 *g* for 1 min. The flow-through was discarded and 0.5 ml QG buffer was added to the column and centrifuged under the same conditions. For washing purpose, 0.75 ml PE buffer was added to the column and centrifuged under the same conditions. The flow-through was discarded again and re-centrifuged under same conditions. To elute DNA, the column was placed onto a new Eppendorf tube and 30 µl TE buffer (2.3.4) was added to the column, left to stand for 1 min and centrifuged under the same conditions. The DNA concentration was measured using an ND-1000 spectrophotometer 'Nanodrop' (Labtech) at λ 260 nm.

2.6.12 PCR Product Purification using PCR Purification Kit

PCR products (2.6.10) were purified when required using QIAquick PCR purification kit (Qiagen). Breifly, PCR product was mixed with buffer PB in a ration of (1:5 V/V), applied to the QIAquick column and centrifuged at 13,000 *g* for 1 min. The flow through was discarded and the column was placed back in the same tube. To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged under the same conditions. After that, the flow-through was discarded and the column was placed back in the same tube and centrifuged for additional 1 min under the same conditions. To elute the DNA, the column was placed into clean Eppendorf tube, 30 µl TE (2.3.4) was added. The column was left to stand for 1 min and centrifuged under the same

conditions. The DNA concentration was measured using an ND-1000 spectrophotometer 'Nanodrop' (Labtech) at λ 260 nm.

2.6.13 Bacteriophage Purification using Caesium Chloride

Selected phage was purified using caesium chloride density gradient as described by Sambrook *et al.*, in 1989. A suspension of the phage containing 10^{11} PFU was mixed with caesium chloride (Sigma-Aldrich) to a final concentration of 0.75 g ml^{-1}, loaded into a Beckman centrifuge tube (Poly allomer tube: quick-seal bell top tubes: 3.5 ml) using a 25 gauge needle and sealed using heat. The tubes were centrifuged at 264,000 *g*, at 4 °C, for 24 h using TLA 100.3 rotor in a Beckman TL-100 Ultra centrifuge. The concentrated phage was indicated by a blue band which was collected by piercing the tube with 23-G hypodermic needle just below the band and withdrawing the solution.

Excess caesium chloride was removed by ultrafiltration using a Microcon 30,000 Da molecular weight cut off column (Millipore, Watford, UK) centrifuged at 6,500 *g* for 10 min in a Biofuge Pico bench-top centrifuge (Kendro Laboratory Products). Followed by washing the pellet twice with 100 µl SM buffer (2.3.1) by centrifugation under the same conditions. The column was then inverted and the phage was eluted in 100 µl SM buffer (2.3.1) by centrifugation under the same conditions and stored at 4 °C until required.

2.7 The Effect of Phage Infection on *Campylobacter* Growth

Fifty ml of NB (2.1.7) was inoculated with an overnight culture of *C. jejuni* PT14 grown on BA (2.1.1) under micoaerobic (2.5.1) conditions at 42 °C that was suspended into 10 ml PBS buffer using a sterile swab.

This experiment was run in three independent replicates and three NB flasks were used as controls. A 1 ml fraction was collected from each flask, diluted 10-fold in PBS (2.3.6) and dispensed using the Miles and Misra technique onto CCDA plates (2.1.2) and incubated at 42 °C for 48 h under microaerobic (2.5.1) conditions.

The cultures were incubated with shaking under microaerobic (2.5.1) conditions for 2 h at 42 °C, 100 rpm, followed by their inoculation with selected phage at a multiplicity of infection (MOI) of one. Samples were collected for bacterial counting on CCDA (2.1.2) plates and phage growth. The incubation was continued for 10 h and samples were collected hourly. Phage counts were performed after storing the samples at 4 °C for 24 h. This was done as described in (2.6.4) on NZCYM (2.1.3) plates.

2.8 Cloning and Expression of Bacteriophage Genes

2.8.1 Competent Cell Preparation using Calcium Chloride ($CaCl_2$) Solution

From overnight cultures incubated at 37 °C of selected *Escherichia coli* strains in LB (2.1.6) containing chloroamphenicol (2.4.2), 4 ml was inoculated in to 400 ml fresh media. All LB incubation was done at 37 °C, shaking at 200 rpm unless otherwise stated. Once the OD at 590 nm reached 0.375, the culture was transferred to ice for 10-15 min. The OD was measured using an Ultra Spec 2000 (UV/Visible) spectrophotometer (GE Health Care, Little Chalfont, UK). The culture then was centrifuged using JA-10 rotor in a J2-21 Beckman centrifuge at 1600 *g* for 10 min at 4 °C without the brake. The pellet was re-suspended in 80 ml of ice cold $CaCl_2$ solution (2.3.10) and kept on ice for 10 min followed by centrifugation 1000 *g* for 5 min at 4 °C without the brake. The pellet was re-suspended in 80 ml ice cold $CaCl_2$ solution (2.3.10) and kept on ice for 30 min. The solution was centrifuged at 1000 *g* for 5 min at 4 °C without the brake. The pellet was resuspended in 8 ml ice cold $CaCl_2$ (2.3.10), dispensed in 100 µl volumes in pre chilled 1.5 ml Eppendorf tubes, frozen using liquid nitrogen and stored at -80 °C until required.

2.8.2 TOPO-Cloning of Bacteriophage CP220 Selected Genes

Selected PCR products (2.6.9) were cloned in pCR 2.1-TOPO using TOPO-Cloning Kit with *E. coli* TOP 10 cells (Invitrogen, Paisley, UK) following the manufacturer description.

The TOPO cloning reaction was set up as follows:

Reagent	Stock Concentration	Final Concentration	Volume Used
PCR Product	Approx. 0.2 μg μl^{-1}	66 ng μl^{-1}	2 μl
Salt solution	1.2 M NaCl 0.6 M MgCL	0.2 M NaCl 0.1 M MgCl	1 μl
TOPO vector	10 ng μl^{-1}	1.67 ng μl^{-1}	1 μl
Water	-	-	2 μl

The reaction mixture was incubated at room temperature (20 °C) for 5 min, then transferred to ice. From the TOPO cloning reaction, 2 μl was taken and added to a vial of chemically competent *E. coli* cells (Invitogen).

2.8.3 Transformation into Chemically Competent Cells

The cloned PCR products (2.8.2) were transferred into a vial of *E. coli* TOP10 chemically competent cells, mixed gently and incubated on ice for 30 min followed by heating shock in a 42 °C water bath for 30 s and then placing back on ice for 2 min. Two hundred and fifty μl of S.O.C medium that had been kept at room temperature was added and transferred to a 15 ml snap-cap tube and shaken at 200 rpm in a 37 °C incubator for 1 h. Aliquots of 20 μl and 10 μl were spread on LB agar plates (2.1.5) containing the appropriate antibiotics. Prior to use if required, these plates were spread with 15 μl of 100 mg ml $^{-1}$ X-Gal (Melford Laboratories Ltd). The plates were incubated at 37 °C for 24 h.

2.8.4 Plasmid Mini Preparation (Using QIA Prep Spin Kit)

From selected overnight cultures of plasmid containing *E. coli* incubated in 10 ml LB containing the appropriate antibiotics at 37 °C for 24 h, aliquots of the cultures obtained were stored in 12.5 % glycerol (2.2.3) at -80 °C and the rest was used for plasmid mini preparation using a QIA prep spin kit (Qiagen) according to the manufacturer's instructions. Briefly, from the culture, 1.5–3 ml was pelleted by centrifugation 13,000 *g* for 1 min using a Biofuge Pico bench-top centrifuge (Kendro Laboratory Products). The pellet was resuspended in 250 µl buffer P1 (50 mM Tris-HCL [pH 8]; 10 mM EDTA; 100 µg ml^{-1} RNase A) followed by the addition of 250 µl buffer P2 (200 mM NaOH; 1 % W/V Sodium dodecyl sulphate). The solution was mixed thoroughly by inverting, after which 350 µl buffer N3 (3 M potassium acetate [pH 5.5]) was added and mixed by inverting. The mixture was centrifuged at 13,000 *g* for 10 min after which the supernatant was applied to the QIA prep spin column and centrifuged at 13,000 *g* for 1 min. The column was washed by adding 0.5 ml buffer BP and centrifuged under the same conditions. The column was washed by adding 0.75 ml buffer PE and centrifuged under the same conditions. The flow through was discarded, and additional centrifugation for one minute was performed before eluting plasmid DNA with 30 µl of TE buffer (2.3.4). Plasmid concentration was measured using ND-1000 spectrophotometer 'Nanodrop' (Labtech) at λ 260 nm and stored at -20 °C for before analysis.

2.8.5 Restriction Digest of the Plasmids in TOPO Vector

Prepared plasmids were digested using *Eco*RI restriction enzyme (Promega) to detect the presence of the inserts.

The reaction mixtures were prepared as follows;

Reagent	Stock Concentration	Final Concentration	Volume Used
Buffer (Promega)	10 X	1 X	2 µl
*Eco*RI (Promega)	10 U μl^{-1}	0.25 U μl^{-1}	0.5 µl
Plasmid	1 µg ml^{-1}	50 ng	1 µl
Sterile, deionized water	-	-	16.5 µl

Following incubation at 37 °C for 1 h, the reaction products were analyzed by agarose gel electrophoresis (2.6.10).

2.8.6 Gene Expression in pET System

The selected vectors and inserts were prepared as follows:

2.8.6.1 Restriction Digest

Samples were grown in LB (2.1.6) containing ampicillin (2.4.1). A portion of the culture was stored in 12.5 % glycerol at -80 °C (2.2.3). The rest was used for plasmid mini preparation (2.8.4).

The restriction digests were performed using *Nde*I (Promega) and *Bam*HI (Promega) as follows:

Reagent	Stock Concentration	Final Concentration	Volume Used
Buffer D (Promega)	10X	1X	2.0 µl
*Nde*I (Promega)	10 U µl^{-1}	0.25 U µl^{-1}	0.5 µl
*Bam*HI (Promega)	10 U µl^{-1}	0.25 U µl^{-1}	0.5 µl
Plasmid	1 µg ml^{-1}	50 ng	1.0 µl
Sterile, deionized water			16.0 µl

After incubation at 37 °C for 1 h, followed by enzyme inactivation at 65 °C for 15 min, the products were examined using a 1 % agarose (Biogene) gel (2.6.10) to establish complete digestion.

2.8.6.2 Vector Dephosphorylation

The digested vector was dephosphorylated using Antractic phosphatase (NewEngland BioLabs LTD, Hitchin, UK) as follows:

Reagent	Concentration Used	Volume Used
Antractic phosphatase buffer 10X	1X	2.0 µl
Digested vector	Approx. (1-5 µg)	17.0 µl
Antractic phosphatase	5 units	1.0 µl

The mixture was then incubated at 37 °C for 15 min, followed by enzyme inactivation at 65 °C for 5 min.

2.8.6.3 Vector and Insert Purification

To purify the dephosphorylated vector and the insert, the samples were run on 0.8 % low melting point agarose (Melford Laboratories LTD, Ipswich, UK) gel (2.6.10) followed by its purification using QIAquick gel extraction kit (Qiagen; 2.6.11).

2.8.6.4 Ligation

The purified insert (2.8.6.3) was ligated with the purified vector (2.8.6.3) using T_4 DNA Ligase (Promega) using a 3:1 molar ratio insert:vector. The reaction mixture was prepared as follows and was then incubated at 16 °C or 16 h.

Reagent	Amount
Vector	100 ng
Insert	"A" ng
Ligase buffer (10 X) (Promega)	1.0 µl
T4 DNA ligase (Promega)	0.1 – 1.0 U
Nuclease free water	Up to 10.0 µl

The molar ratio can be calculated using the following formula: [(100 ng vector * kb insert) / kb vector) * (3/1)] = "A" ng insert

2.8.6.5 Transformation into a non-Expression Host

The ligated mix was first dialyzed for 20 min using 0.025 μm VSWP Millipore filters, and then it was transformed into a non expression host TOP 10 chemically competent *E. coli* cells (Invitrogen) as described in 2.8.3.

2.8.6.6 Transformation into Expression Host

Plasmids from confirmed positive clones were transformed into *E. coli* expression hosts that were prepared (2.8.1) to allow protein expression as described in 2.8.7.

2.8.7 Test Protein Expression using the pET System

Colonies obtained from (2.8.6.6) were inoculated into 10 ml LB (2.1.6) containing ampicillin (2.4.1) and chloroamphenicol (2.4.2). Part of the culture was stored in 12.5 % glycerol (2.2.3) and the rest was used in the experiment.

To 20 ml of fresh media, 1 ml culture was added and incubated at 37 °C, shaking at 200 rpm until the OD 600 nm reached 0.3-0.4. After this time a 1ml sample was taken as "pre–induced" sample for total cell extraction and the rest of the sample was induced with 0.1 mM IPTG (2.3.8), and incubated. After 4 h, a sample (1 ml) was taken, from which the OD 600 was measured using an Ultra spec 2000 (UV/Visible) spectrophotometer (GE Health Care) and a total cell protein extract prepared as follows.

The sample was centrifuged at 13,000 *g* for 1 min in Biofuge Pico bench-top centrifuge (Kendro Laboratory Products). The supernatant was discarded and

the pellet was resuspended in nuclease free water, for which the volume was calculated as follows: Water Volume = OD600 * 100.

The extracted fractions were stored at -20 °C for analysis.

2.8.8 Isolation of Material from Cell Fractions

From an overnight culture of the sample expressing the protein, 1 ml was transferred to 20 ml LB (2.1.6) containing ampicillin (2.4.1) and chloroamphenicol (2.4.2), and incubated until the OD 600 reached 0.3-0.4 (measured using Ultra spec 2000 [UV/Visible] spectrophotometer [GE Health Care]). After which a 1 ml sample was taken as a "pre-induced" sample and the rest of the sample was induced with 0.1 mM IPTG (2.1.8), incubated for 4 h and another 1 ml sample was taken as the "post-induced" sample.

Fractions were prepared as follows: The sample was centrifuged at 13,000 *g* for 1 min in Biofuge Pico bench-top centrifuge (Kendro Laboratory Products). The supernatant was discarded and the pellet was re-suspended in cell lysis solution (2.3.12) containing 1 mg ml^{-1} lysozyme (2.3.9), and incubated on ice for 10 min, followed by its centrifugation at 13,000 *g* for 1 min. The supernatant was collected as the "periplasmic" fraction. The pellet was re-suspended in 100 mM Tris-Cl (2.3.13), frozen in liquid nitrogen and thawed in 37 °C water bath. This step was repeated twice, before the samples were centrifuged at 13,000 *g* for 5 min. The supernatant was collected as the "cytoplasm" fraction and the pellet was re-suspended in 1 % triton-X-100

(2.3.14), kept at 4 °C for 10 min and retained as "membrane proteins and inclusion bodies" fraction.

For the pre-induced samples, 100 µl aliquots were used whereas for the post induced samples 200 µl aliquots were used for protein analysis.

The extracted fractions were stored at -20 °C before analysis.

2.8.9 Effect of Induction with IPTG on Cell Growth

From an overnight culture of protein expressing *E. coli* (2.8.6.6) a 1 ml sample was transferred to a 20 ml LB (2.1.6) containing ampicillin (2.4.1) and chloroamphenicol (2.4.2) and incubated for a further 12 h. The OD 600 was measured hourly using Ultra spec 2000 (UV/Visible) spectrophotometer (GE Health Care). When the OD 600 nm reached 0.3-0.4, the samples were induced with 0.1 mM IPTG (2.3.8) and their incubation continued for 12 h.

2.8.10 Electrophoresis Using Tricine Gels

The protein profiles of recombinant *E. coli* extracts (2.8.7, 2.8.8) were visualized by electrophoresis on 10-20 % Novex tricine gels using a propriatary XCell *sureLock* mini cell system (Invitrogen). The sample to be run was prepared by mixing the protein extracts (5 µl) with 2 X Novex Tricine SDS sample buffer (Invitrogen; final concentration 50 % v/v); 10 X NuPAGE reducing agent (Invitrogen; final concentration 10 % v/v) and made up with nuclease free water to give a final volume of 20 µl. Followed by heating the samples at 85 °C for 2 min, and then centrifuged at 13,000 *g* for 1 min. The

samples were loaded on the gel with a See Blue Plus 2 pre-stained standard (Invitrogen) and the gels electrophoresed using 1 X running buffer (Invitrogen) for 90 min at a constant 125 V. At the end of the run, the gels were stained (2.3.15) for 30 min and de-stained (2.3.16) for 1 h.

2.9 Mass Spectrometry

Mass spectrometry (MS) work was performed as a service by R. Susan Liddle at the University of Nottingham using the following procedures.

2.9.1 Gel Piece Processing and Tryptic Digest

Samples to be digested were prepared on 10-20 % Tricine gels (2.8.10) after which they were stained using colloidal coomassie blue (Fisher) and de-stained in water. The target samples were excised from gels using a sterile scalpel followed by their processing in gel pieces using the ProteomeWorks MassPREP robotic liquid handling station (Waters, Elstree, UK). Such samples were incubated in 100 µl of de-stain solution (50 mM ammonium bicarbonate, 50 % acetonitrile) for 10 min at room temperature and which was repeated three times. The final aliquot was dehydrated by incubation in 50 µl of acetonitrile for 5 min at room temperature. Then, the acetonitrile was removed and the gel plugs were for 10 min to allow evaporation. The sample was then incubated for 30 min in 50 µl of reducing solution (10 mM dithiothreitol, 100 mM ammonium bicarbonate) which was removed at the end of the incubation period. The samples were incubated for 20 min in 50 µl of alkylation solution (55 mM iodoacetamide, 100 mM ammonium bicarbonate) at room temperature. Gel slices washing was carried out at room temperature in the

following solution; 50 µl of 100 mM ammonium bicarbonate for 10 min, 50 µl of acetonitrile for 5 min and dehydrated by double room temperature washes in 50 µl of acetonitrile for 5 min and evaporation for 5 min. The microtitre plate containing the gel plugs was cooled to 6 °C for 10 min before the addition of 25 µl per well of trypsin gold (Promega) and diluted to 10 ng μl^{-1} in trypsin digestion buffer (50 mM ammonium bicarbonate). Trypsin entry into the gel plugs was allowed with minimal autocatalysis by incubating the plate at 6 °C for 20 min and at 40 °C for 4 h. Samples were stored at 4 °C until MS analysis.

2.9.2 Mass Spectrometry Analysis of Samples

Samples analysis was carried out by LC-ESI-tandemMS on a Q-TOFII mass spectrometer fitted with a nanoflow ESI (electrospray ionization) source (Waters Ltd). Peptides were separated on a PepMap C18 reverse phase, 75 µm i.d., 15-cm column (LC Packings) and delivered on-line to the MS via a CapLC HPLC system. The mass spectrometer was operated with a capillary voltage of 3000 V in positive ion mode. Argon was used as the collision gas. Acquiring the tandem MS data was achieved using automated data-dependent switching between MS and MS/MS scanning which depends on the ion intensity, mass and charge state (data directed analysis (DDA^{TM}). For such experiments the methods were created in the MassLynx 4.0 software where the charge state recognition was used to select doubly, triply and quadruply charged precursor peptide ions for fragmentation. For the tandem MS acquisition, one precursor mass was selected at a time. The collision energy was selected automatically

according to the charge and mass of each precursor and varied from 15 to 55 eV.

2.9.3 *De novo* Sequence Interpretation of Tryptic Peptides

A *de novo* sequence interpretation for individual peptides was performed using the PepSeq tool of the MassLynx™ 4.0 software package (Waters). TandemMS spectra from the DDA LC-tandemMS runs were processed into singly charged, mono-isotopic masses using MaxEnt 3 maximum entropy software (Waters). The resulting spectra were opened in the PepSeq window, and combinations of automated and manual direction of the tools were used to elucidate each peptide sequence.

The *de novo* peptide sequences were used to perform database searches using BLASTP (at http://www.ncbi.nih.gov) using parameters for "short, nearly exact matches".

2.10 His Tag Addition to the Expressed Protein

Oligonucleotides were designed to insert at the 5'-end of the gene to create an N-terminal fusion with the expressed protein. The oligonucleotides were purchased from MWG (Germany) and kinased using T4 polynucleotide kinase (Promega) following the manufacturer's recommendations.

Briefly, the reaction mix was prepared as indicated below and incubated at 37 °C for 30 min.

Reagent	Stock Concentration	Final Concentration	Volume Used
Oligonucleotides	100 pmol	2.5 pmol	1 µl
Kinase buffer	10 X	1 X	4 µl
ATP	10 mM	1 mM	4 µl
T4 Polynucleotide Kinase	10 U μl^{-1}	0.375 U μl^{-1}	1.5 µl
Water	-	-	29.5 µl

The reaction was stopped by the addition of 2 µl 0.5 M EDTA (2.3.7) and 1 volume of phenol:chloroform:isoamylalcohol (25:24:1; Sigma), before vortexing for 1 min and centrifugation in a bench top centrifuge operating at 13,000 *g* for 2 min. The upper aqueous layer was collected and 1 volume of chloroform:isoamylalcohol (24:1; Sigma) was added, vortexed and centrifuged as described earlier. Following centrifugation, the upper layer was collected and transferred to clean Eppendorf and 0.5 volume of 7.5 M ammonium acetate (2.3.11), 2 volumes 100 % ethanol (Fisher) added and mixed. The sample was left at -80 °C for 30 min, and then centrifuged for 5 min. Finally, the supernatant was discarded and the pellet resuspended in 20 µl TE (2.3.4).

The kinased forward and reverse oligonucleotides were mixed together and heated at 90 °C for 3 min. The Eppendorf tube containing the mix was left in the boiling water in a double ice box and allowed to cool slowly over night. Followed by their self-ligation using T_4 DNA ligase (Promaga) prepared by mixing and incubating the annealed oligonucleotides at 25 °C for 3 h, followed by heat deactivation at 65 °C for 15 min.

Reagent	Stock Concentration	Final Concentration	Volume Used
Oligonucleotides	-	-	12 µl
Ligase buffer	10 X	1 X	1.5 µl
T4 DNA Ligase (Promega)	10 U μl^{-1}	1 U μl^{-1}	1.5 µl

The ligation was first dialyzed for 20 min using 0.025 µm VSWP Millipore filters, and then digested using *Nde*I at 37 °C for 1 h before deactivation at 65 °C for 15 min. The pET3a vector containing the gene to which the His-tag was to be appended was digested with *Nde*I and dephosphorylated (2.8.6.2).

Reagent	Stock Concentration	Final Concentration	Amount
Sample	-	-	15 µl
Buffer D (10X)	10 X	1 X	2.0 µl
Nde I (Promega)	10 U μl^{-1}	0.67 U μl^{-1}	2 µl
Water	-	-	11 µl

The His tag oligonucleotides were ligated into the vector using T4 DNA ligase (Promega) as described in 2.8.6.4, followed by transformation into *E.coli* TOP10 chemically competent cells (2.8.3).

2.11 Lytic Activity of Expressed Proteins

Bacterial lawns of various bacterial strains were prepared as described in 2.6.1, to which protein samples were applied to the lawns as replicate 10 µl droplets. The droplets were allowed to absorb into the agar and all the plates were either incubated at 42 °C under microaerobic conditions (2.5.1) or aerobically at 37 °C for 24 h.

2.12 Trichloroacetic Acid (TCA) Precipitation

Purified phage preparations were subject to TCA precipitation prior electrophoresis on 10-20 % Novex Tricine gels (2.8.10) for MS analysis. Briefly, to CsCl density gradient purified phage CPX, a one tenth volume of 0.15 % deoxycholate (Sigma-Aldrich) was added, vortexed and incubated for 10 min, after which a one tenth volume of 72 % TCA was added and incubated for a further 5 min at room temperature. The solution then was centrifuged at 13,000 *g* in a Pico bench-top centrifuge (Kendro Laboratory Products) for 8 min. The supernatant was removed using a needle and syringe and the pellet was resuspended in 20 µl SM buffer (2.3.1).

CHAPTER THREE

ISOLATION OF CAMPYLOBACTERS AND BACTERIOPHAGES

3.1 Introduction

Campylobacter jejuni is considered the most important cause of bacterial foodborne gastroenteritis worldwide (Reid *et al.*, 2008). Campylobacters are ubiquitous in nature with a large environmental reservoir residing in poultry, where they form part of the commensal microflora (Beery *et al.*, 1988; Newell and Fearnley 2003). Organic and free-range farming practices have been shown to produce birds with a higher incidence of *Campylobacter* colonisation (Heuer *et al.*, 2001; El-Shibiny *et al.*, 2005). In one study on raw chicken meat sold in the UK, 80% was contaminated with *Campylobacter* (Jørgensen *et al.*, 2002) with 98% of isolates speciated as *C. jejuni* and 2% *C. coli*. The probability of isolating phages is increased where their hosts thrive, so poultry, in particular free range chickens, are a likely source of phages (Atterbury *et al.*, 2003a). The work in this chapter describes the isolation of *Campylobacter* and their phages from free-range layer chickens exposed to the environment. This was carried out to extend the availability of characterised phages for use in phage therapy against campylobacters in domestic poultry (Loc Carrillo *et al.*, 2005).

3.2 Results

3.2.1 *Campylobacter* and Bacteriophage Isolation, Propagation, Concentration and Titration

Fresh chicken faeces collected from free range chickens from three different farms in Leicestershire were transported to the laboratory in cool boxes within 30 minutes of collection and screened immediately for the presence of campylobacters and phages. The results are listed in Table 3.1. *Campylobacter* isolation was performed by direct plating on CCDA plates (see section 2.5.4) and confirmed by Gram stain (see section 2.5.3) to be Gram-negative. *Campylobacter* isolates were stored in *Campylobacter* storage medium (2.2.1). Campylobacters were isolated from 51 % of the total number of samples (35). Of the samples collected from Heath Farm, 57 % were *Campylobacter* positive (8/14), and from Calcutt Heights, 50 % of the samples were positive (10/20). Lastly, the sample obtained from Minster veterinary practice was negative.

All the phages were isolated and propagated from the samples according to the method described previously in sections 2.6.2 and 2.6.3. Phages were successfully isolated from Heath Farm and Calcutt Heights Farm (Table 3.1), and these coincided with the *Campylobacter*-positive samples at the respective frequencies of 14 % (2/14) and 5 % (1/20). The phages were numbered 3b, 4a (Heath Farm) and 18b (Calcutt Heights Farm). Phage 3b proved to be difficult to propagate in the laboratory. However, the remaining two phages were plaque purified, concentrated (see section 2.6.3) and titrated (see section 2.6.4)

on *C. jejuni* PT14. Their titre was found to be was 10^9 PFU ml^{-1} for each of them.

Table 3.1 Isolation of Campylobacters and bacteriophages

	Farm Name			
	Heath Farm	**Calcutt Heights**	**Minster**	**Total**
Number of Samples	14	20	1	35
Samples Positive for *Campylobacter*	8	10	0	18
Samples Positive for phages	2	1	0	3

3.2.2 Susceptibility of Campylobacters to Bacteriophages

The ability of the three phages isolated in the previous section to produce plaques on range of campylobacters was examined. This analysis was extended to include some previously characterized and uncharacterized phage in order to compare their lytic profiles. The uncharacterized phages were recovered from retail chicken and were of interest because they had been demonstrated to possess larger DNA genomes (W phages; Atterbury *et al.*, 2003a) on the basis of estimates from pulsed fields gel electrophoresis. The lytic profiles of all the phages were compared using a number of *Campylobacter* strains from different sources; broiler, human and reference sources including strains previously found to be resistant to phage attack.

Campylobacter strains were obtained from various different sources: Health Protection Agency, Queens Medical Centre and the Laboratory collection from broiler and layer chickens are listed in Table 3.2. Bacterial lawns were prepared from each strain as described in section 2.6.1. Filtrates recovered

from environmental suspensions (4a and 18b) as described previously in section 2.6.2, and a set of laboratory phage isolates (Table 3.3) initially recovered from retail chicken; (W2, W3, W5, W7, W8; Atterbury *et al.*, 2003a), were applied to bacterial lawns prepared (see section 2.6.1) as 10 µl aliquots containing 10^6 or 10^9 PFU ml^{-1} to examine their lytic effects on these strains. Table 3.4 presents the results of susceptibility testing of *Campylobacter* strains against various phages.

Bacterial lawns were tested with 10^9 PFU phages to intentionally overload them in order to look for minority populations of mutant phage with different host ranges to the majority of the population. However, if the strain was sensitive to the majority of the phage, the results were generally not different except in the case of HPC5 and OCIIC10, which produced better defined zones of lysis on the host bacterial lawns compared to the routine test dilution of 10^6 PFU that resulted in no lysis of OCIIC10 or semi-confluent lysis for HPC5 with phage 4a only.

All the phages tested had the same lytic effect on C. *jejuni* PT5 and PT33 resulting in complete lysis of the lawn. None of the phages were virulent against NCTC PT1, PT2, PT35, 12668 (Health Protection Agency); 163,166 (QMC); MPVC1, T1VC9, GIIC8, OR12 and OCIIIC2 (Laboratory stocks) with no plaques formed even on the overloaded spots. Phage 18b was the only phage found to lyse *Campylobacter* human strains 162 and 164.

Table 3.2 Details of *Campylobacter* strains used in this chapter

All strains are *C. jejuni* except: NCTC 12668, OR12 and PT44 are *C. coli*

Strain	Source	Isolated from	Year obtained
PT14	HPA Colindale	Human faeces	2000
PT1	HPA Colindale	Human faeces	2003
PT2	HPA Colindale	Human faeces	2003
PT5	HPA Colindale	Human faeces	2003
PT16	HPA Colindale	Human faeces	2003
PT19	HPA Colindale	Human faeces	2003
PT33	HPA Colindale	Human faeces	2003
PT35	HPA Colindale	Human faeces	2003
PT44	HPA Colindale	Human faeces	2003
11168	NCTC	Human faeces	2000
12668	NCTC	Human faeces	2000
161	Queen Medical Centre	Human faeces	2005
162	Queen Medical Centre	Human faeces	2005
163	Queen Medical Centre	Human faeces	2005
164	Queen Medical Centre	Human faeces	2005
165	Queen Medical Centre	Human faeces	2005
166	Queen Medical Centre	Human faeces	2005
MPVC1	Laboratory stock	Broiler chicken cloacal swab	2001
T1VC9	Laboratory stock	Broiler chicken cloacal swab	2001
GIIC8	Laboratory stock	Broiler chicken cloacal swab	2001
OR12	Laboratory stock	Free range chicken excreta	2007
HPC5	Laboratory stock	Broiler chicken cloacal swab	2001
FDC3	Laboratory stock	Broiler chicken cloacal swab	2001
DV1FS	Laboratory stock	Broiler chicken cloacal swab	2001
OCIIIC2	Laboratory stock	Broiler chicken cloacal swab	2001

Table 3.2 cont. Details of *Campylobacter* strains used in this chapter

All strains are *C. jejuni* except: NCTC 12668, OR12 and PT44 are *C. coli*

Strain	Source	Isolated from	Year obtained
OCIIC10	Laboratory stock	Broiler chicken cloacal swab	2001
HP I C10	Laboratory stock	Broiler chicken cloacal swab	2001
OK VIII C3	Laboratory stock	Broiler chicken cloacal swab	2001
HP I F9	Laboratory stock	Broiler chicken excreta	2001
BIII C7	Laboratory stock	Broiler chicken cloacal swab	2001
OC I F3	Laboratory stock	Broiler chicken excreta	2001
G III F2	Laboratory stock	Broiler chicken excreta	2001
TIIIC9	Laboratory stock	Broiler chicken cloacal swab	2001
CIIC3	Laboratory stock	Broiler chicken cloacal swab	2001
TIF2	Laboratory stock	Broiler chicken excreta	2001
FD I C2	Laboratory stock	Broiler chicken cloacal swab	2001
OK IX C3	Laboratory stock	Broiler chicken cloacal swab	2001
OK X C2	Laboratory stock	Broiler chicken cloacal swab	2001
ES 1	Laboratory stock	Free range chicken excreta	2001
GHII F7	Laboratory stock	Broiler chicken excreta	2001
C I C4	Laboratory stock	Broiler chicken cloacal swab	2001
DV IV C1	Laboratory stock	Broiler chicken cloacal swab	2001
DV IVC8	Laboratory stock	Broiler chicken cloacal swab	2001
GHIC10	Laboratory stock	Broiler chicken cloacal swab	2001
MPV C1	Laboratory stock	Broiler chicken cloacal swab	2001
B103032A	The University of Bristol	Broiler chicken house doors (swab)	2008
B103066A	The University of Bristol	Broiler chicken house excreta	2008
B10309B	The University of Bristol	Broiler chicken house overshoes	2008
B103037A	The University of Bristol	Broiler chicken house anteroom overshoes	2008

Table 3.2 cont. Details of *Campylobacter* strains used in this chapter

All strains are *C. jejuni* except: NCTC 12668, OR12 and PT44 are *C. coli*

Strain	Source	Isolated from	Year obtained
B103025A	The University of Bristol	Broiler chicken house overshoes	2008
B10269	The University of Bristol	Broiler chicken house overshoes	2008
B102632B	The University of Bristol	Broiler chicken house (swab back double doors)	2008
B102635C	The University of Bristol	Broiler chicken house (swab anteroom door)	2008
B10C61B	The University of Bristol	Broiler chicken house excreta	2008
C1235100B	The University of Bristol	Broiler chicken caecal contents	2008
C124136C	The University of Bristol	Broiler chicken house (swab anteroom equip.)	2008
C123566c	The University of Bristol	Broiler chicken house excreta	2008
C1241103B	The University of Bristol	Broiler chicken caecal contents	2008
C122514B	The University of Bristol	Broiler chicken house (swab outside grass)	2008
C1235105A	The University of Bristol	Broiler chicken caecal contents	2008
D15C9C	The University of Bristol	Broiler house (swab concrete outside)	2008
D15C83B	The University of Bristol	Broiler house swab	2008
D15C18C	The University of Bristol	Broiler chicken house (swab outside grass)	2008
D15C10B	The University of Bristol	Broiler house (swab concrete outside)	2008
D15C82A	The University of Bristol	Broiler house swab caecal contents	2008
D15C46B	The University of Bristol	Broiler chicken house (outside puddle)	2008
D15C17B	The University of Bristol	Broiler chicken house (swab outside grass)	2008
D15C2	The University of Bristol	Broiler chicken house (swab outside grass)	2008

Table 3.3 Details of bacteriophages used in this chapter

Phage	*Campylobacter* propagation strain	Source	Isolated from	Year
CP8	PT14	Laboratory stock	Free Range chicken excreta	2001
CP20	FDC3	Laboratory stock	Broiler chicken excreta	2001
CP30	PT14	Laboratory stock	Broiler chicken excreta	2001
CP34	PT14	Laboratory stock	Broiler chicken excreta	2001
CP220	PT14	Laboratory stock	Broiler chicken carcass	2003
CPX	PT14	Laboratory stock	Broiler chicken carcass	2007
W2	PT14	Laboratory stock	Poultry meat	2001
W3	PT14	Laboratory stock	Poultry meat	2001
W5	PT14	Laboratory stock	Poultry meat	2001
W7	PT14	Laboratory stock	Poultry meat	2001
W8	PT14	Laboratory stock	Poultry meat	2001
3b	PT14	This thesis	Free range chicken excreta	2007
4a	PT14	This thesis	Free range chicken excreta	2007
18b	PT14	This thesis	Free range chicken excreta	2007

Table 3.4 Susceptibility of *Campylobacter* strains to bacteriophages

Campylobacter strains from 1-11 were obtained from HPA Colindale, strains 12-17 were human isolates obtained from QMC; strains 18-26, were from laboratory stocks. CL, confluent lysis; SCL, semi-confluent lysis; -, no plaques were formed.

Results were obtained using 10^6 and 10^9 PFU for all strains. Where there was no difference between the two different concentrations, only the 10^6 PFU phage concentration is presented. Where there was a difference between the two concentrations both results are presented.

	Campylobacter Strains	Bacteriophages						
		W2	W3	W5	W7	W8	4a	18b
1	PT14	CL	CL	CL	SCL	CL	SCL	CL
2	PT1	-	-	-	-	-	-	-
3	PT2	-	-	-	-	-	-	-
4	PT5	CL	CL	CL	CL	CL	CL	CL
5	PT16	-	SCL	-	CL	-	-	SCL
6	PT19	-	CL	-	-	CL	SCL	-
7	PT33	CL	CL	CL	CL	CL	CL	CL
8	PT35	-	-	-	-	-	-	-
9	PT44	-	-	-	-	-	SCL	-
10	11168	SCL	-	-	-	-	-	SCL
11	12668	-	-	-	-	-	-	-
12	161	SCL	-	-	SCL	-	SCL	SCL
13	162	-	-	-	-	-	-	SCL
14	163	-	-	-	-	-	-	-
15	164	-	-	-	-	-	-	SCL
16	165	SCL	-	-	SCL	-	-	SCL
17	166	-	-	-	-	-	-	-
18	MPVC1	-	-	-	-	-	-	-
19	T1VC9	-	-	-	-	-	-	-
20	GIIC8	-	-	-	-	-	-	-
21	OR12	-	-	-	-	-	-	-
22	FDC3	SCL	CL	-	CL	CL	SCL	SCL
23	DV1FS	-	-	SCL	-	-	-	-
24	OCIIIC2	-	-	-	-	-	-	-
25	HPC5 with 10^6 PFU	-	-	-	-	-	SCL	-
	with 10^9 PFU	-	CL	CL	-	CL	CL	-
26	OCIIC10							
	with 10^6 PFU	-	-	-	-	-	-	-
	with 10^9 PFU	CL	CL	CL	-	CL	-	-

To extend this comparison a further set of *Campylobacter* strains were tested (Table 3.2) using laboratory stock phages: CP8, CP20, CP30, CP34, CP220 (Loc Carrillo *et al.*, 2005; Scott *et al.*, 2007; El Shibiny *et al.*, 2009) and CPX, which was co-isolated with CP220 (listed in Table 3.3). The bacterial lawns were prepared from each strain as described in section 2.6.1, and the phages propagated and titrated according to the methods described in sections 2.6.3 and 2.6.4. The phages were then applied to the lawns as 10 µl spots containing with 10^6 PFU to examine their lytic effects on these strains to enable comparison with the first set of strains. The phage lytic profiles are shown in Table 3.5.

All phages could be discriminated on the basis of their lytic profiles. None of the tested phages were virulent against the following strains; PT1, OR12, GHIIF7, CIC4, DVIVC1, DVIVC8, GHIC10, MPVC1, B10269, C124136C, C1241103B and C122514B. PT2 was lysed only by phage CP220, FDIC2 by CPX and OKIXC3, OKXC2 and ES1 only by CP20. *Campylobacter* strains HPC5, HPIC10, D15C9C and D15C17B were lysed by all tested phages.

Table 3.5 Susceptibility of *Campylobacter* strains to bacteriophages (10^6 PFU)

CL, confluent lysis; SCL, semi-confluent lysis; OL, opaque lysis -, no plaques were formed.

	Campylobacter Strains	Bacteriophages					
		CP8	CPX	CP20	CP30	CP34	CP220
1	PT1	-	-	-	-	-	-
2	PT2	-	-	-	-	-	OL
3	PT44	CL	CL	CL	-	-	CL
4	OR12	-	-	-	-	-	-
5	TIVC9	-	-	CL	CL	-	-
6	HPC5	CL	CL	CL	CL	CL	SCL
7	OCIIIC2	-	-	-	-	-	-
8	12668	-	-	CL	-	-	CL
9	HP I C10	CL	SCL	CL	CL	CL	CL
10	OK VIII C3	SCL	-	SCL	CL	CL	-
11	HP I F9	CL	-	CL	CL	CL	OL
12	BIII C7	-	-	-	CL	CL	-
13	OC I F3	-	-	OL	CL	SCL	-
14	G III F2	-	-	OL	OL	OL	-
15	TIIIC9	-	-	-	CL	CL	-
16	CIIC3	-	CL	CL	-	-	-
17	TIF2	-	-	CL	-	-	OL
18	FDIC2	-	SCL	-	-	-	-
19	OKIXC3	-	-	CL	-	-	-
20	OKXC2	-	-	CL	-	-	-
21	ES1	-	-	OL	-	-	-
22	GHIIF7	-	-	-	-	-	-
23	CIC4	-	-	-	-	-	-
24	DVIVC1	-	-	-	-	-	-
25	DVIVC8	-	-	-	-	-	-
26	GHIC10	-	-	-	-	-	-
27	MPVC1	-	-	-	-	-	-
28	B103032A	-	-	-	SCL	SCL	-
29	B103066A	-	-	-	CL	SCL	-
30	B10309B	-	-	-	OL	-	-
31	B103037A	-	-	-	CL	CL	-
32	B103025A	-	-	CL	-	-	CL
33	B10269	-	-	-	-	-	-
34	B102632B	-	-	-	CL	CL	-
35	B102635C	-	-	-	CL	CL	-
36	B10C61B	-	-	CL	-	-	CL
37	C1235100B	-	-	-	CL	CL	-
38	C124136C	-	-	-	-	-	-
39	C123566c	-	-	CL	CL	CL	CL
40	C1241103B	-	-	-	-	-	-
41	C122514B	-	-	-	-	-	-
42	C1235105A	-	-	-	CL	CL	-
43	D15C9C	CL	CL	CL	CL	CL	CL
44	D15C83B	-	-	CL	-	-	SCL
45	D15C18C	-	-	CL	OL	OL	-
46	D15C10B	OL	-	CL	CL	CL	OL
47	D15C82A	-	-	CL	-	-	CL
48	D15C46B	CL	-	CL	CL	CL	CL
49	D15C17B	CL	CL	CL	CL	CL	CL
50	D15C2	-	-	CL	OL	-	-

3.2.3 Pulsed Field Gel Electrophoresis (PFGE)

To estimate the genomic DNA sizes of the newly isolated phages 3b, 4a, 18b, and other phages from the laboratory stocks including: W2, W3, W5 and W7, samples were prepared in agarose blocks for PFGE as described in section 2.6.7, and following electrophoresis the gels were stained with ethidium bromide and the images captured with a ChemiDoc XRS Imager using the Quantity one program (Bio-Rad).

Gels revealed that phage preparations of 3b, 4a, 18b, W2, W3, and W7 contained a discreet a single DNA band corresponding to the genomes of these phages. The sizes of the newly isolated phages; 3b, 4a and 18b were estimated at 145 kb whilst the W2, W3 and W7 phages sizes were estimated to be 180, 194 and 195 kb respectively. In contrast two bands were obtained from W5, a major band of estimated size 130 kb and a minor band of 195 kb (Figure 3.1).

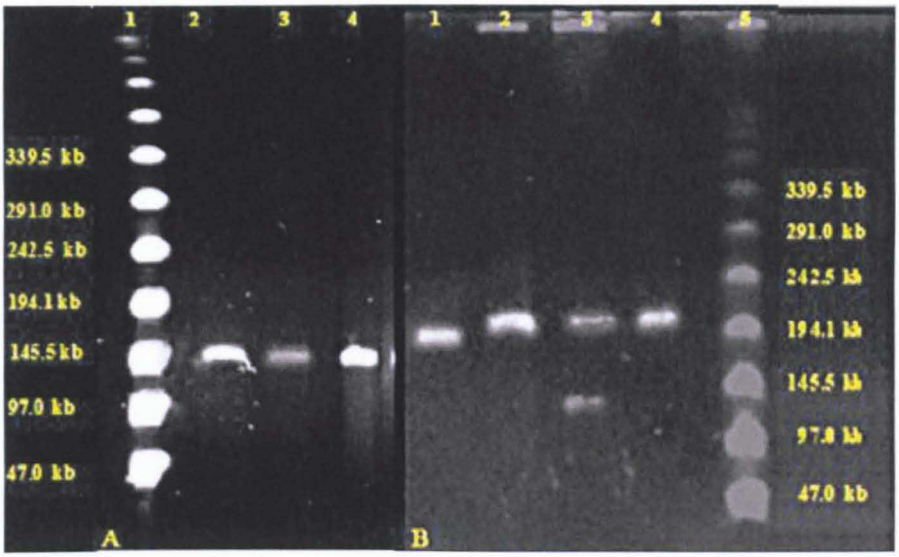


Figure 3.1 PFGE showing genomic DNA of selected bacteriophages

In order to determine the genomic size of different phages, samples were prepared and run using PFGE. Lanes represented are as follows;

A) Lane 1; PFGE DNA marker, Lane 2; phage 4a, Lane 3; phage 3b, Lane 4; phage 18b

B) Lane 1; phage W2, Lane 2; phage W3, Lane 3; phage W5, Lane 4; phage W7, Lane 5; PFGE DNA marker

3.2.4 Separation of W5 bands Obtained from PFGE

The phage W5 was found to have two different sized DNA bands by PFGE (Figure 3.1). In order to find whether these DNAs represented two forms of the same phage or two different phages in the same sample, attempts were made to separate them by plaque purification.

Ten-fold serial dilutions of phage W5 at 10^8 PFU ml^{-1} were prepared and the 10^{-5}, 10^{-6}, 10^{-7} dilutions were mixed with 500 µl *C. jejuni* PT14 (Universal propagation strain) suspension that was prepared by collecting the PT14

growth from blood plate using a swab and suspending it in 10 mM $MgSO_4$ (described in section 2.3.2). The selected dilutions were propagated as described in section 2.6.3. After plaque formation, 10 well-isolated plaques from the 10^{-7} dilution, were picked using 1000 µl Gilson tips and transferred to Eppendorf tubes containing 500 µl SM buffer (described in section 2.3.1), and each left overnight at 4 °C under shaking conditions. Propagation of the phage was repeated using 200 µl of these suspensions (undiluted) under similar conditions but this time each plaque was propagated on five plates. After incubation, 5 ml of SM buffer (described in section 2.3.1) was added to each plate and kept at 4 °C overnight with gentle shaking. The phage suspension was then collected, concentrated and titrated as described earlier in sections 2.6.3 and 2.6.4. PFGE blocks were then prepared to liberate genomic DNAs and a PFGE gel was prepared and run as described in section 2.6.7.

The PFGE image is shown in Figure 3.2. One sample (lane 6) did not show any visible DNA bands. Two samples (lanes 3 and 9) appeared as a smear and the rest of the samples revealed a DNA bands of equivalent size to the smaller genomic DNA band (130 kb) observed in the preparation from the original plaque isolate.

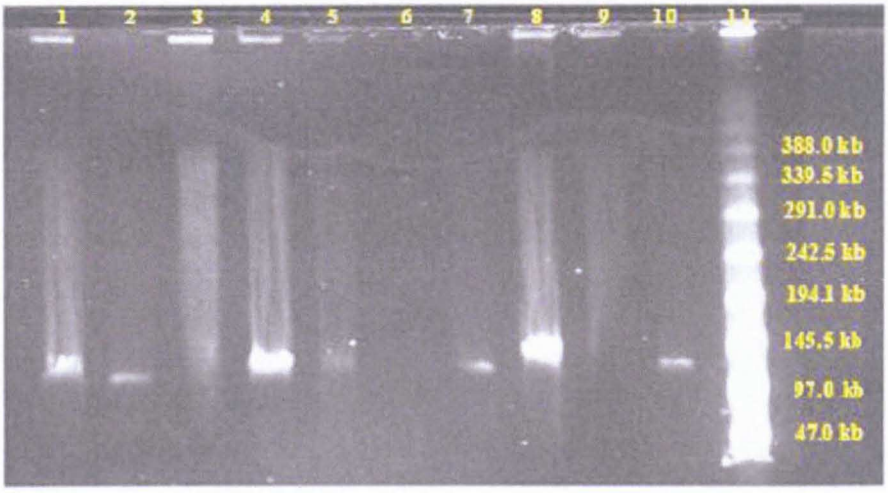


Figure 3.2 PFGE of genomic DNA of different plaques from W5 phage

PFGE of phage W5 revealed two DNA bands of different sizes. In order to determine whether these fragments belonged to the same phage, attempts were made to plaque purify them, from which samples were prepared for separation of the genomic DNAs by PFGE. Lanes represented as follows;

Lanes 1 to 10; genomic DNA from 10 different plaques of W5 phage, Lane 11; PFGE DNA marker

3.3 Discussion

Campylobacters were isolated from 51 % of the chicken faecal samples examined in this chapter with around 8 % also containing *Campylobacter* phages. A high prevalence of *Campylobacter* in free-range chicken has been reported previously (Rivoal *et al.*, 2005). All the phages tested could be distinguished from each other based on their lysis profiles with host *Campylobacter* strains. The phage diversity encountered could arise from the acquisition of phage from multiple environmental sources, or alternatively once acquired, the phage could adapt within the chicken intestinal tract to maximize the use of the host campylobacters available to them. Samples which were negative for campylobacters were negative for phages as well. This correlates with the finding that phages are present only when their hosts are present. Since poultry in UK are frequently colonized by campylobacters, the high percentage of their isolation was expected (Corry and Atabay, 2001).

Phages were isolated from the samples analysed at a frequency of 8 %, which was somewhat lower than the frequency observed by Atterbury *et al.* (2005) of approximately 45 %, but from the data presented by Connerton *et al.* (2004), the frequency of isolation is variable, depending on whether a suitable sensitive host is available. The isolated phages were plaque purified, and propagated in order to determine their genomic sizes. PFGE can be used to separate large DNA fragments efficiently (McCelland *et al.*, 1987), and it has been used for genomic investigations of different strains of bacteria and phages (Prevots *et al.*, 1990; Loc Carillo *et al*, 2007). Genomic DNAs of the isolated phages 3b, 4a and 18b were estimated at 145 kb in size, which places them in group III

according to the classification system of Sails *et al.* (1998), who classified phages into three classes based on their average size into groups I, II and III with average sizes of 320, 184 and 138 kb, respectively. According to the genomic size estimates of the W phage series obtained from PFGE, they can be placed into group II based on Sails *et al.* (1998).

Phage preparations of W5 produced two DNA fragments on the PFGE gel indicating that two different phages might be present. Obviously, when characterizing a phage isolate it is important that it is a pure culture, so experiments were performed in order try to separate them. However, these efforts only resulted in the isolation of the smaller phage at the expense of the larger phage or in one case, another example of the two genome sizes remaining together. The smaller phage was of a genome DNA size typical of group III phages, and was probably recovered more frequently because the population of this phage in the sample was greater than the larger one. It was apparent from the original PFGE gel (Figure 3.1) that the genome concentration was lower for the larger sized phage. To be able to investigate whether the two DNA bands on the PFGE gel resulted from the same phage with two variations in size or two completely different phages, more work would need to be done using larger scale preparations in conjunction with DNA sequencing. The observations regarding W5 were similar to those regarding CP220 (see Chapter 5), in that in some preparations two DNA bands were visible. In the latter case it was possible to separate the preparation into two phages with different sized genomes, CP220 and CPX. As the sequence of

CP220 and was available, efforts were concentrated on obtaining the sequence of its co-isolate CPX (Chapter 5) rather than W5.

All the phages isolated in our laboratory could be classified into groups II and III (Atterbury *et al.*, 2003a; Connerton *et al.*, 2004). Group I phages were not isolated, which is likely to be because Group I phages are either not common in the UK or in the *Campylobacter* sources sampled (chickens and humans).

The lytic profiles of all the phages studied in this chapter were distinguishable from each other using a diverse set of *Campylobacter* strains, which highlights the diversity in *Campylobacter* phage specificity. None of the phages lysed the laboratory strains MPVC1, T1VC91, GIIC8, OR12, OCIIIC2, GHIIF7, CIC4, DVIVC1, DVIVC8 and GHIC10 that had been specifically selected as being more resistant to phage attack on the basis of previous screening with different phage sets (Dr. P. Connerton, University of Nottingham, pers. Comm.), and highlights that more work needs to be done in order to find suitable phages that might be used to treat such strains in phage therapy.

Bacterial strains can be resistant to phage treatments. One possible reason for such resistance is the interaction between the phages and host receptors and co-receptors. Phage attachment to its host is the first step in phage infection and any change in the surface profile either by regulated expression or mutation in the receptors will lead to phage insensitivity. For that reason, understanding phage receptors is required to optimize phage cocktails that can be used for bacterial control and food spoilage prevention (Shin *et al.*, 2012). In addition to that, infection level can be reduced in cells having DNA restriction

modification systems by destroying foreign DNA and host cell genes leading to phage replication block (Rees and Loessner, 2009).

It was hoped that by testing a concentration of the phages (10^9 PFU ml^{-1}), that contained a much higher number of phage than would normally be required for lytic profiling, that variants within the phage population effective against these problematic strains might be detected. However, in this instance no variants were detected. The standard dose used for lytic profiling (10^6 PFU ml^{-1}) allowed the host ranges of the different phage to be ascertained and the results were generally easier to read than those in which excess phage titres were added. These strains remain insensitive to the bacteriophage but it is unclear at this time whether this is because they do not bind or are prevented from completing their life cycle.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF CP220 BACTERIOPHAGE AND RELATED BACTERIOPHAGES

4.1 Introduction

The complete genome sequence of phage CP220 was determined by workers at the University of Nottingham and the Sanger Centre (Timms *et al.*, 2010). The availability of this data allowed the phage CP220 sequence to be used as the template on which primers could be designed for DNA amplification for the characterization and analysis of other phages. Some regions of particular interest included the putative lysin genes (CPT 0075, 120 and 142), because of their potential antimicrobial action. The lysin genes were the first selected for characterization, followed by a gene with a possible transposase function (CPT 0035c) together with structural genes (CPT 0034c, 0052, 0053, 0045, 0058, 0175).

CPt10 is another phage in the same class as CP220, the genomic sequence of which was published together with that of CP220. The two sequences exhibit a great deal of similarity. In order to investigate the relationships between these phages and those from other classes, a number of genes were selected within the CPt10 genome sequence for amplification. These included a lipoprotein gene (CPt10_ 0771), a head completion protein (CPt10_0591), a methyl transferase gene (CPt10_1471) and a transposase gene (CPt10_1761). PCR would then be used to find out if they were present in CP220 and other phages.

4.2 Results

4.2.1 Propagation and Concentration of Bacteriophages

A number of phages were selected for this study including CP220, CP8, CPX, CP20, CP30 and CP34. The phages were propagated (see section 2.6.3) and titrated (see section 2.6.4) using the C. *jejuni* host strain PT14 with the exception with CP20, which was titrated on C. *jejuni* HPC5. The number of plaque forming units (PFU) was calculated and found to be 10^9 for each phage preparation.

4.2.2 DNA preparation and Pre-amplification of Bacteriophages

Genomic DNA from phages: CP220, CPX, CP8, CP20, CP30 and CP34 were prepared according to the method described in section 2.6.8. The concentration of the prepared DNA was insufficient to perform all the molecular charactization experiments. Thus, DNA of all phages was pre-amplified using whole genome amplification REPLI-g kit as described in section 2.6.9, which resulted in samples with higher DNA concentrations for their use in subsequent experiments.

4.2.3 Gradient Polymerase Chain Reaction Amplification of Genes from Bacteriophage CP220

Three genes from CP220 phage were selected (CPT 0075, 120 and 142) for PCR amplification. Primers (Eurofins MWG Operon, Ebersberg, Germany) were designed and used for amplification of each gene. The primer sequences are listed in Table 4.1

Table 4.1 Primer sequences of bacteriophage CP220 potential lysin genes

CP220 Gene	Position	Sequence
CPT 0075	68295…68588	F: 5'-TTC ATA TGC CTC CTT TAA CTA GAG TTG GTG-3' R: 5'-TTG GAT CCT TAT CCA CAA ATA ACA TTT CCA GAT CC-3'
CPT 120	107127… 107801	F: 5'-AAC ATA TGA ATT ACG ATA AAC TGA ATA AAA TGG G-3' R: 5'- AAG GAT CCG TAT TAA GAG TTT AGT TTA TTA AAT TTT GAC-3'
CPT 142	126849… 127019	F: 5'-TTC ATA TGA ATA ATT CTG GAG TTT GTT CAT TTC-3' R: 5'-TTG GATCCC TAT GTT TTT GCA AGA CAA GAA ACC C-3'

In order to determine the optimum annealing temperature for DNA amplification, gradient amplification using a range of temperatures was carried out for the selected genes from phage CP220 in C1000tm thermal cycler (Biorad), using the reaction mixture described in methods section 2.6.10 using the following cycles.

CPT 0075, CPT 120 and CPT 142		
1 cycle	96°C	3 min
35 cycles	96°C 55-65°C 72°C	1 min 1 min 1 min
1 cycle	72°C	7 min

At the end of the program cycle, a 1 % agarose gel (see methods section 2.6.11) was run using 20 % of the PCR products together with 1 kb DNA markers (Promega), and the gels examined and the image recorded with a

ChemiDoc XRS Imager using the Quantity one program (Bio-Rad). Figure 4.1 presents the agarose gel of the gradient PCR amplification products.

The annealing temperatures tested were: 55.8, 57.4, 59, 60.6 and 62.2 °C The optimum annealing temperature for CP220 CPT 120 was established to be between 60.6 and 62.2 °C (Figure 4.1A), which was the same for CP220 CPT0075 and 142 (Figure 4.1B). By analyzing these results, 61 °C was chosen as the annealing temperature for further PCR amplification regimes.

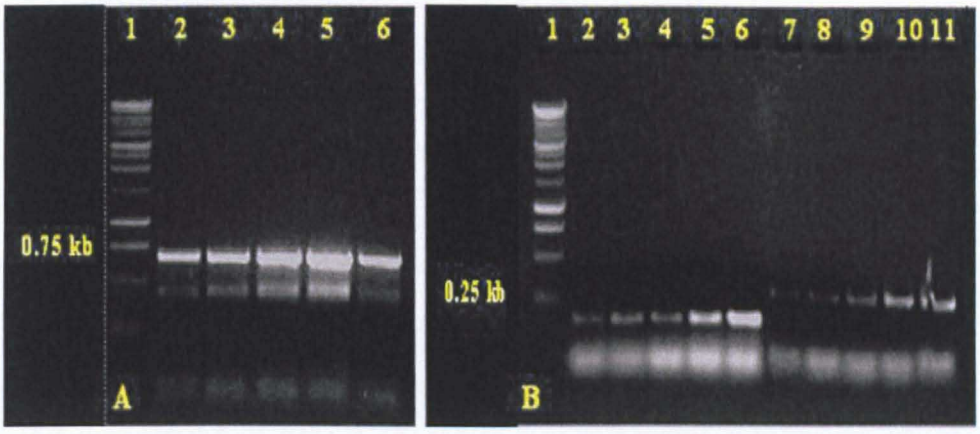


Figure 4.1 Gradient PCR amplification of phage CP220 selected genes

In order to determine the optimum annealing temperature for phage CP220 DNA amplification, gradient PCRs were run using primers designed for the amplification of the selected genes. PCR products were examined using 1 % agarose gel electrophoresis. Lanes are represented as follows:

A; CPT 120

Lane 1; 1 kb DNA marker, Lanes 2-6; PCR products at temperatures 55.8, 57.4, 59, 60.6 and 62.2 °C

B; CPT 142 and 0075

Lane 1; 1 kb DNA marker, Lanes 2-6; PCR products at temperatures 55.8, 57.4, 59, 60.6 and 62.2 °C for CPT 142, Lanes 7-11; PCR products at temperatures 55.8, 57.4, 59, 60.6 and 62.2 °C for CPT 0075

4.2.4 Amplification of Potential Lysin Genes From Bacteriophage CP220

The annealing temperature for PCR amplification was selected to be 61 °C. Primers (Table 4.1) were designed to amplify the potential lysin genes CPT 0075, 142 and 120 from phage CP220 DNA (accession number: FN667788). Phage CP220 DNA, prepared as described in methods section 2.6.8, was amplified using the reaction mixture as described in section 2.6.10 using a TC-312 thermal cycler (Scientific Laboratory Supplies) programmed with the following cycles:

1 cycle	96℃	3 min
35 cycles	96℃ 61℃ 72℃	1 min 1 min 1 min
1 cycle	72℃	7 min

The PCR products were examined using 1 % agarose gel electrophoresis (see section 2.6.11) together with a 100 bp DNA marker (Promega). The gel image was recorded with a ChemiDoc XRS Imager using the Quantity one program (Bio-Rad), and is shown in Figure 4.2. The DNA fragment sizes were estimated as follows: CPT 120 fragment was 664 bp whereas the fragment sizes of CPT 142 and CPT 0075 were 204 and 302 bp, respectively.

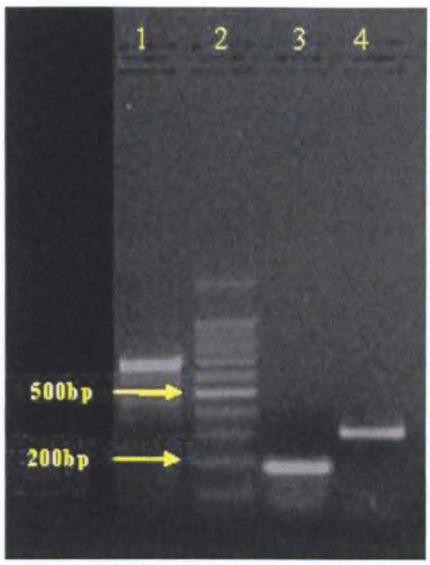


Figure 4.2 PCR amplification of phage CP220 potential lysin genes

Phage CP220 DNA was amplified using primers designed for the potential lysin genes. The PCR products were examined by 1 % agarose gel electrophoresis and the fragment sizes estimated to be 664, 204 and 302 bp for CPT 120, CPT 142 and CPT 0075, respectively. Lanes representing 20 % of the PCR amplification products are as follows: Lane 1; CPT 120, lane 2; 100 bp DNA marker, lane 3; CPT142, lane 4; CPT 0075.

4.2.5 Class II *Campylobacter* Bacteriophage Genes in Other Bacteriophages

In order to compare any relationship between different phages, DNA from selected phages (CPX, CP8, CP20, CP30, CP34 and CP220) isolated previously from chicken sources were prepared (see methods section 2.6.8). The phage DNAs were used as templates for PCR amplification (see methods section 2.6.10) with primers (Eurofins MWG Operon) specifically designed upon selected genes from the Class II bacteriophage genomes of CP220 and CPt10 available in the database (Tables 4.1 and 4.2). The gene selection was

based on their potential function (for instance lysins, transposases, lipoproteins) or because their presence had been examined in phages of the UK *Campylobacter* typing scheme as part of another study performed by Timms *et al.* (2010), in this case the GP18 and GP19 structural genes

The amplification was carried out using reaction mixture in detailed in section 2.6.10 using TC-312 thermal cycler (Scientific Laboratory Supplies) using the following cycles:

1 cycle	96℃	3 min
35 cycles	96℃ 61℃ 72℃	1 min 1 min 1 min
1 cycle	72℃	7 min

The PCR products were examined using a 1 or 1.5 % agarose gels loaded with 1 kb and/or 100 bp DNA markers (Promega). The gel image was recorded with a ChemiDoc XRS Imager using the Quantity one program (Bio-Rad). The DNA fragments were extracted and purified from agarose gel slices as described in section 2.6.13 and DNA sequenced using the Euorofins MWG Operon value one service. The DNA sequences obtained were analyzed using the BioEdit program. The nucleotide sequence similarities and differences between the amplified DNAs and the class II bacteriophage genes are listed in Appendices 1 and 2.

Table 4.2 Primer sequences of selected Class II bacteriophages genes

CP220 phage genes		
Genes	**Position**	**Sequence**
CPT 0035c	C*27555...28799	F: 5'- GGT GGA TCA AAA GCA ACA GG -3' R: 5'-AGA GGA AAG ATT TGC GAC CA-3'
GP18 CPT 0034c	C*25935...27485	F: 5'- GGG TAT GCA AGT TTG CCA AG -3' R: 5'- AAG CAC AAC AAT CAC CAC GA -3'
GP18 CPT 0052	48293...49909	F: 5'- AGA TCC AAG CCA AGC AAG TG -3' R: 5'- TGG CCT TGA ACC TTC TTT TG -3'
GP18 CPT 0053	49913...51649	F: 5'- CGA TCG CGT AAA CGT TGT TA -3' R: 5'- TAC GCT TGC TGG ATT GTT TG -3'
GP19 CPT 0045	41505...42263	F: 5'- CAA GTT TTC CGG GAT TGA CT -3' R: 5'- TCC TCA AAT TGA TGT GCT TGA -3'
GP19 CPT 0058	56719...57309	F: 5'- ACC CAG GGG GCT TAT TTA CA -3' R: 5'- GCC GAT TTA CCA TCT TGT GG -3'
GP19 CPT 175	157455...158207	F: 5'- AAG ATG CGA GGT GAA ACA GA -3' R: 5'- CCG CTT TCA AAG TTC CGT TA -3'
CPt10 phage genes		
Genes	**Position**	**Sequence**
CPt10_0591	59093...60181	F: 5'- AGC CGA GCT GCT AAA CTG AG -3' R: 5'- GAA AAG CGT TGC CCT TGT AT -3'
CPt10_0771	68741...69277	F: 5'- TTC TTG GAA TGT TGG AAA TGT T -3' R: 5'- AGC TTT GAA TGG GTT TGC TG -3'
CPt10_1471	123774...124439	F: 5'- TCC AAA TCG TGT TTT GTT ATG G -3' R: 5'- TTT TTG TGT TGG GTG AAC GA -3'
CPt10_1761	147098...148342	F: 5'- GAT CAA AAG CAA CAG GCT TAC A -3' R: 5'- TCT CCT CCG TCT TAG AGG AAA -3'

* C used to indicate that this gene is located on the complementary strand

Primers sequences obtained from Timms and others (2010)

4.2.5.1 Comparison to CP220 Bacteriophage

The CP220 phage genes included a number of potential lysin genes (CPT 0075, 120 and 142), a gene with possible transposase function (CPT 0035c) and a number of GP18 (CPT 0034c, 0052 and 0053) and GP19 (CPT 0045, 0058 and 0175) structural genes.

Comparison of the amplicon sequences to phage CP220 DNA sequences indicated that the putative lysin genes (CPT 0075, 120 and 142) were amplified successfully from the genomic DNAs of phages CPX, CP8, CP20, CP30 and CP34. The amplified DNA fragments for these genes are presented in Figures 4.3, 4.4 and 4.5 respectively. In addition, CPT 0035c (Figure 4.6) with a possible transposase function, and structural genes corresponding to phage CP220 proteins: GP18 (CPT 0034c, 0052 and 0053) and GP19 (CPT 0045, 0058 and 0175) respectively were also amplified and the DNA sequences compared (Figures 4.7 and 4.8). The DNA fragments of the genes that were successfully PCR amplified (Table 4.3) were gel purified (see methods section 2.6.11) and the DNAs sequenced. The sequences obtained were analyzed using BioEdit and appear in Appendix 1.

Table 4.3 Bacteriophage PCR amplification with CP220 gene specific primers

Amplified Gene	Bacteriophage				
	CPX	CP8	CP20	CP30	CP34
CPT 0075	+	+	+	+	+
CPT 120	+	+	+	+	+
CPT 142	+	+	+	+	+
CPT 0035c	-	-	+	-	-
GP18 CPTs; 0034c, 0052, 0053	-	-	+	-	-
GP19 CPTs; 0045, 0058,0175	-	-	+	-	-

+= Successful amplification of the expected DNA product

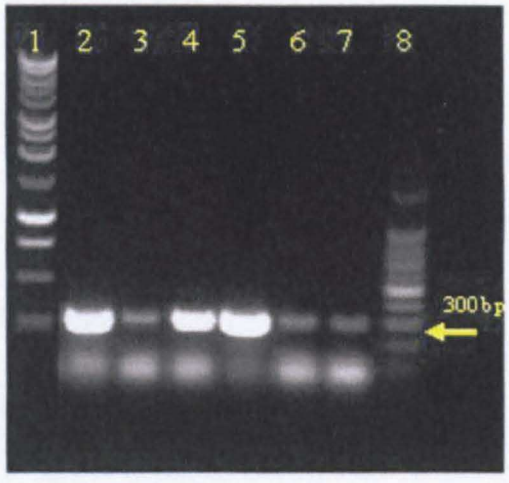


Figure 4.3 Phage CP220 CPT 0075 gene in other phages

In order to detect the presence of the CP220 gene CPT 0075 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lane 1; 1 kb marker, lane 2; CP220, lane 3; CPX, lane 4; CP8, lane 5; CP20, lane 6; CP30, lane 7; CP34, lane 8; 100 bp marker.

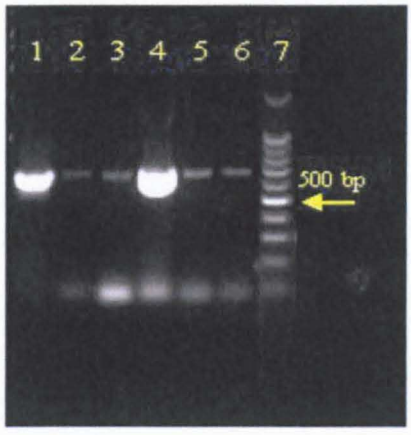


Figure 4.4 Phage CP220 CPT 120 gene in other phages

In order to detect the presence of the CP220 gene CPT 0120 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lane 1; CP220, lane 2; CPX, lane 3; CP8, lane 4; CP20, lane 5; CP30, lane 6; CP34, lane 7; 100 bp marker.

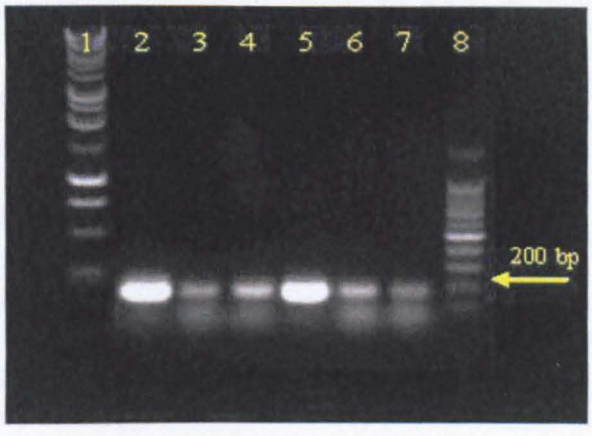


Figure 4.5 Phage CP220 CPT 142 gene in other phages

In order to detect the presence of the CP220 gene CPT 00142 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers The PCR products were examined using 1 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lane 1; 1 Kb marker, lane 2; CP220, lane 3; CPX, lane 4; CP8, lane 5; CP20, lane 6; CP30, lane 7; CP34, lane 8; 100 bp marker.

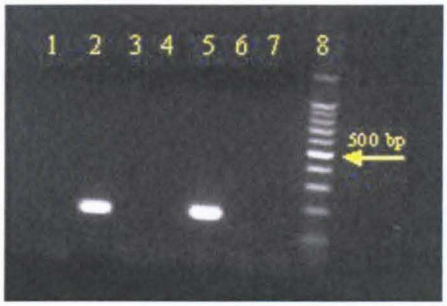


Figure 4.6 Phage CP220 0035c gene in other phages

In order to detect the presence of the CP220 gene CPT 0035c in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1.5 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lane 1; negative control, lane 2; CP220,lane 3; CPX, lane 4; CP8, lane 5; CP20, lane 6; CP30, lane 7; CP34, lane 8, 100 bp marker.

Figure 4.7 Phage CP220 GP18 genes in other phages

In order to detect the presence of CP220 GP18 alleles 0034c, 0052 and 0053 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1.5 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lanes 1 to 7; CP220 CPT 0034c, lanes 8 to14; CP220 CPT 0052, lanes 15 to 21; CP220 CPT 0053 and lane 22; 100 bp Marker. The order of the samples in lanes 1 to 7, 8 to 14 and 15 to 21 are negative control, CP220, CPX, CP8, CP20, CP30 and CP34, respectively.

Figure 4.8 Phage CP220 GP19 genes in other phages

In order to detect the presence of CP220 GP19 alleles 0045, 0058 and 0175 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1% agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lanes 1 to 7; CP220 CPT 0045, lanes 8 to14; CP220 CPT 0058, lanes 15 to 21; CP220 CPT 0175 and lane 22; 100 bp Marker. The order of the samples in lanes 1 to 7, 8 to 14 and 15 to 21 are negative control, CP220, CPX, CP8, CP20, CP30 and CP34, respectively.

The sequences obtained from DNA amplifications using the 0075 primers were identical to CP220 CPT 0075 for phages CPX, CP8, CP30 and CP34. CP20 however, had 14 nucleotide differences at the following positions: A42T, C54T, G67A, G93A, A132G, G142C, G143C, C153T, G156A, G174A, C182T, T207C, C252G and A264G), which translated to four amino acid differences (V23I, I44M, G48P and A61V). Phages amplified with the CPT 120 primers showed identity with CP220 CPT 120 for CP8 and CP30. CPX showed three nucleotide differences (T72C, T92C, T445C) that translate to two amino acid differences (V31A, F149L). CP20 showed 21 nucleotide differences (A46G, T64C, A150G, T154C, A219G, T235A, A257G, A270G, C278A, A327G, C366T, T375C, G408A,A414G, T417G, T435C, T447C,

G465T, T468C, G525A, T568C) that translate to 7 amino acid differences (I16V, F22L, S79T, K86R, A93D, K155N, S190P).The DNA sequence of the CPT120 of CP34 was incomplete when compared with CP220. The CPT 120 PCR product lacked 132 bp from the end of the gene and contained the following substitutions within the conserved region (A33C, A109T, C268 and A492C). CPX, CP20, CP30 and CP34 showed 100 % identity to CP220 CPT 142 whereas CP8 showed two nucleotide differences (G36A, C108T) that are silent.

CP20 was the only phage that could be amplified using the 0035c primers. The sequences showed two nucleotide differences (G181A, T222C) translating to one amino acid difference (V81A) compared to CP220 CPT 0035c.

Amplification of genomic DNA with primers designed to amplify parts of several structural genes was carried out. CP20 was the only phage DNA that could be amplified with the GP18 (CPT 0034c, 0052 and 0053) and GP19 (CPT 0045, 0058 and 0175) primers. The CP20 sequence showed 100 % identity to CP220 CPT 0052, 0053 but exhibited eight nucleotide differences (A772G, A775G, C777T, G800A, T804C, T817C, G823A, G825A) that translate to four amino acid differences (T258A, T259A, S267N, V275I) with CP220 CPT 0034c. Comparison with CP20 GP19 genes showed the following changes: two nucleotide differences (A258C, A261T) for CPT 0045, one nucleotide difference (A525G) for CPT 0058 and four nucleotide differences (T252C, C288T, G303A, T312C) for CPT 0175 compared to CP220. These differences in nucleotides were silent and did not lead to any change in the protein sequences.

4.2.5.2 Comparison to CPt10 Bacteriophage

Bacteriophage CPt10 is another group II *Campylobacter* phage that shows close sequence homology to phage CP220 for which the DNA sequence (accession number: FN667789) is available for use in sequence comparison studies. To extend the analysis, four genes were selected with the gene annotations indicating the following potential functions: head completion protein gene (CPt10_0591), lipoprotein gene (CPt10_0771), methyl transferase (CPt10_1471) and a transposase (CPt10_1761).

The positive DNA amplification products are listed in Table 4.6 and the amplified products are shown in Figures 4.9 and 4.10. These DNA fragments were purified as described in methods section 2.6.13, and sequenced using the Eurofins MWG Operon value one service. DNA sequences were analyzed using the BioEdit program and appear in Appendix 2.

Table 4.4 Bacteriophage PCR amplification with CPt10 gene specific primers

Amplified Gene	Bacteriophage					
	CP220	CPX	CP8	CP20	CP30	CP34
CPt10_0771	-	-	-	+	-	-
CPt10_0591	-	+	-	+	-	-
CPt10_1471	-	+	-	+	-	-
CPt10_1761	+	+	-	-	-	-

+= Successful amplification of the expected DNA product

Figure 4.9 Phage CPt10 (0771, 0591) genes in other phages

In order to detect the presence of selected CPt10 genes 0071 and 0591 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1.5 % agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows: Lanes 1 to 6 show amplification reaction products with CPt10 gene-specific primers to CPt10_0771, lanes 7 to12 shows amplification reaction products with CPt10 gene-specific primers to CPt10_0591 and lane 13 100 bp marker. The order of the samples in lanes 1 to 6 and 7 to 12 are negative control, CP220, CPX, CP8, CP20, CP30 and CP34, respectively.

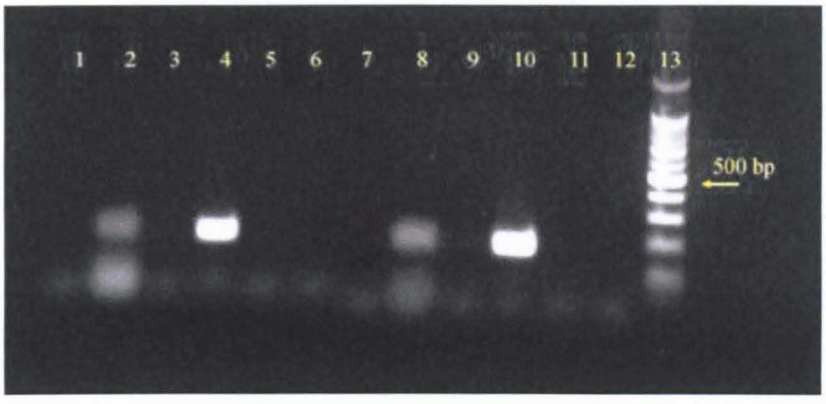


Figure 4.10 Phage CPt10 (1471, 1761) genes in other phages

In order to detect the presence of selected CPt10 genes 1471 and 1761 in alternative phage genomes, their DNAs were PCR amplified using gene-specific primers. The PCR products were examined using 1.5% agarose gel electrophoresis. Lanes representing 20 % of the amplified DNA fragments from the different phages are as follows:

Lanes 1 to 6 show amplification reaction products with CPt10 gene-specific primers to CPt10_1471, lanes 7 to12 show amplification reaction products with CPt10 gene-specific primers to CPt10_1761 and lane 13; 100 bp Marker. The order of the samples in lanes 1 to 6 and 7 to 12 are negative control, CP220, CPX, CP8, CP20, CP30 and CP34, respectively.

CP20 was the only phage DNA that could be amplified with CPt10_0771 primers, the DNA sequence of which showed 100 % identity to CPt10_0771 in the amplified region. Phages CPX and CP220 were amplified using the 1761 primers with 100 % identity for phage CPX, with 5 nucleotide differences were recorded for phage CP220 (C222T, C242T, A311T, A313G, T314C), which led to three amino acid differences (A81V, D104V, I105A). Phages CPX and CP20 were amplified using the CPt10_0591 and 1471 primers. The amplified products showed 100 % identity to the original phage CPt10 DNA sequence.

4.2.6 PFGE of Bacteriophage DNAs

In order to estimate the sizes of the DNA genomes from phages CP220, CPX, CP8, CP30 and CP34, phage DNAs were prepared in agarose blocks and subjected to pulsed fields gel electrophoresis (PFGE) calibrated with phage lambda DNA marker as described in methods section 2.6.7.

PFGE of phage DNA preparations CPX, CP8, CP30 and CP34 revealed one band corresponding to a genome size of 145.5 kb (Figure 4.11). However, the genomic DNA sizes of CP20 and CP220 were estimated at 194 Kb.

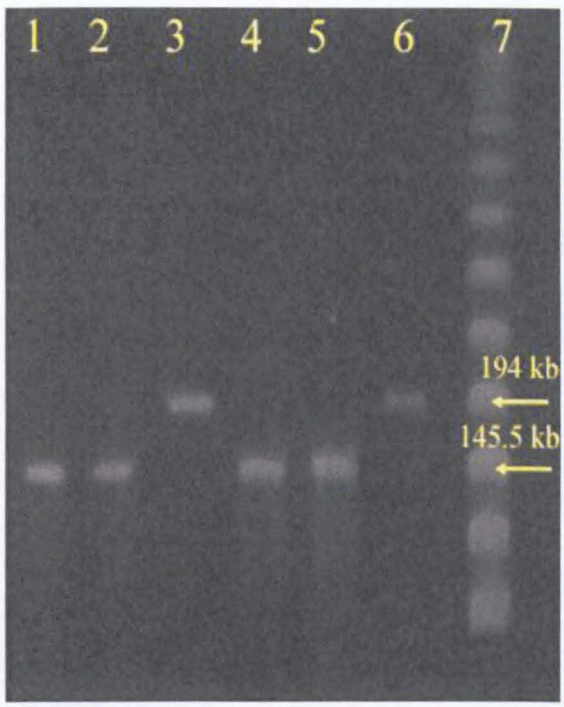


Figure 4.11 PFGE of genomic DNAs of selected *Campylobacter* phages
In order to determine the genome sizes of *Campylobacter* phages, their DNAs were prepared and subject to PFGE. Gel lanes were loaded as follows: Lane 1; CPX, lane 2; CP8, lane 3; CP20, lane 4; CP30, lane 5; CP34, lane 6; CP220, lane 7; DNA marker (concatenated phage lambda DNA).

4.3 Discussion

The work in this chapter was undertaken in order to characterize the potential lysin genes from phage CP220 and determine if they were conserved in other phages. In addition a transposase encoding gene and several structural genes were investigated together with selected genes from phage CPt10. Potential lysin genes were amplified from CP220 and the other phage, and their sequences compared. These and other genes were used to examine the relationships between the different phages with respect to the PCR amplification products from these genes.

The absence of DNA amplification does not always indicate the absence of the gene, where situations such assequence divergence or DNA modification can prevent PCR amplification (Timms *et al.*, 2010).

Primers designed from CP220 genes were then used to amplify DNA prepared from the phages CPX, CP8, CP20, CP30 and CP34. Comparison of the PCR amplicons from these phages with CP220 showed that CP20 is more closely related to CP220 than any of the other phages selected. The same phages were also compared to phage CPt10, which is similar to CP220 and revealed that for the selected genes CPX appeared to be more closely related to CP220 and CP20 than the other phage tested. This was surprising because CPX is a group III phage and CP220, CPt10 and CP20 belong to group II. This is intriguing when considering that the reason for characterizing CPX was because of its intimate relationship with CP220.

Genes that could have lytic activities (CPT 0075 and 120) were found in all the phages tested. Further studies involving expression and characterization of the protein products of these genes could potentially lead to the development of novel antimicrobial products. Equally, the product of the transposase gene could potentially result in development of a useful biotechnological tool, and provides evidence for the widespread presence of the transposase. The sequences of the structural genes allow different phages to be compared. The PCR primers designed for this study will be useful in further studies to monitor the presence of phage, for example in intervention trials. They may allow simple discrimination between different phages used together in a phage cocktail, in order to follow the intervention process through the chicken intestine.

CHAPTER FIVE

CPX BACTERIOPHAGE

5.1 Introduction

Genome sequencing is an important step towards understanding how phages function at the molecular level. Three T-even phages (T2, T4 and T6) discovered between 1920 and 1960 have been used as model systems to identify genes and genetic mechanisms of phage biology, which have provided insights into fundamental mechanisms applicable in higher organisms (Cairns *et al.,* 1992). Among these phages, T2 was the first to be isolated and used in many studies until the mid 50's when T4 became preeminent. T4 was notably used in two studies by Benzer (1962) exploring the structure of two phage genes and in a collaborative study to collect and characterise T4 conditional lethal mutants (Epstein *et al.,* 1963; Edgar 2004). The outcome of their studies led to T4 becoming the reference phage or prototype for its relatives (Petrov *et al.,* 2010). Recently, examples of *Campylobacter* phage genome sequences that potentially fall within the T4-like group have been reported, these include phages: CP220, Cpt10 (Timms *et al.,* 2010), CP81 (Hammerl *et al.,* 2011), NCTC12673 (Kropinski *et al.,* 2011), vB_CcoM-IBB_35 (Carvalho *et al.,* 2012) and CP21 (Hammerl *et al.*, 2012).

The work in this chapter describes the purification, characterization and complete genome sequence of the phage known as CPX. This particular phage was of interest because it was recovered as a co-isolate of phage CP220 that was sequenced by Timms *et al.* (2010) from poultry carcass based on the observation that PFGE of phage genomic DNA preparations produced two bands, indicating the presence of a phage DNA of 220 kb with that of a 140 kb DNA despite plaque purification. The larger genome corresponds to the class II

phage CP220 and the smaller with the more frequently occurring class III phage of 140 kb. Whilst it was difficult to separate the co-existing phages, CPX was eventually separated from CP220 by picking multiple individual plaques and checking each for a single 140 kb or 220 kb genome by PFGE. The nucleotide sequence of phage CP220 genome was recently reported (Timms *et al.*, 2010). A study of the sequence and host range of CPX was then undertaken to determine if it had any relationship to CP220 in order to investigate if there was any significance in their co-existence and also to generate more sequence data to increase our knowledge of *Campylobacter* phages.

5.2 Results

5.2.1 Transmission Electron Microscopy of Bacteriophage CPX

The phage CPX was propagated in the laboratory as described in methods section 2.6.3 and treated with ammonium acetate as described in methods section 2.6.5 before examination by electron microscopy. The phage preparation was titrated (see methods section 2.6.4) and found to contain 10^8 PFU.

Sample preparation and electron microscopy was performed by Dr. S. Hyman at Leicester University. Briefly, phage particles were absorbed onto a glow-discharged carbon-coated Pioloform grids and fixed for 2 min by exposure to vapour from a 25 % v/v glutaraldehyde solution. After washing, the grids were

negatively stained using 0.5 % w/v uranyl acetate, followed by their examination using a JEOL 100CX transmission electron microscope operating at an acceleration voltage of 80 kV.

Examination of electron micrographs (Figure 5.1) revealed that CPX was a typical *Campylobacter* phage of the family *Myoviridae* with an icosahedral head measuring 101 nm and tail measuring 105 nm.

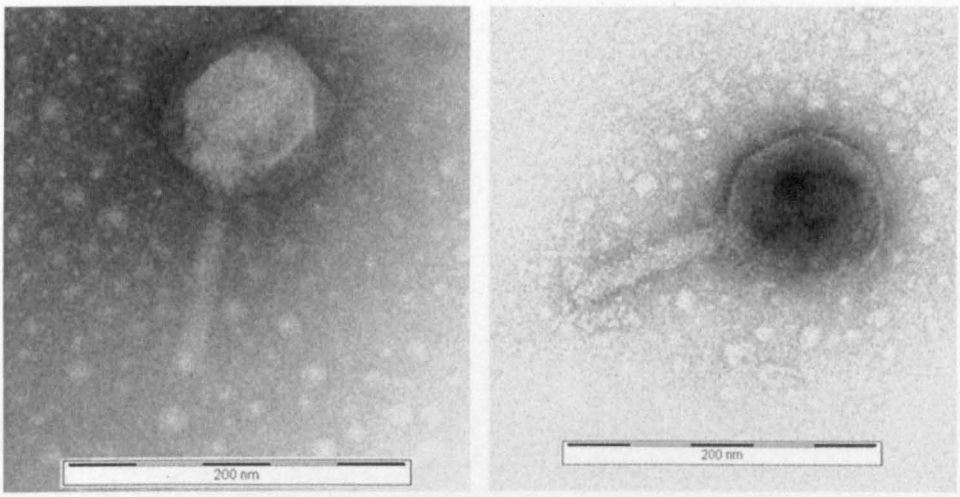


Figure 5.1 Transmission electron micrographs of CPX bacteriophage

5.2.2 CPX Purification Using Caesium Chloride Density Gradient Centrifugation

The CPX phage was propagated on lawns of *C. jejuni* PT14, concentrated and titrated as described in sections 2.6.3 and 2.6.4 to produce a phage preparation containing 10^{11} PFU ml^{-1}. This phage preparation was mixed with caesium chloride (CsCl) to a final concentration of 0.75 g ml^{-1} CsCl, loaded into 3.5 ml polyallomer tubes and centrifuged at 264,000 *g*, at 4 °C, for 24 h using a TLA

100.3 rotor in Beckman TL-100 Ultracentrifuge (see methods section 2.6.13). Following ultracentrifugation of CPX to form a caesium chloride density gradient two blue bands were clearly visible (Figure 5.2). Each band was carefully collected (200 µl) for further analysis following dialysis.

Excess caesium chloride was removed by centrifugation using a Microcon 30,000 Da molecular weight cut off filter (Millipore, Watford, UK) at 6,500 *g* for 10 minutes in a Biofuge Pico bench-top centrifuge (Kendro Laboratory Products). The retentate was washed twice with 100 µl SM buffer (see section 2.3.1), before the column was inverted and CPX recovered in 100 µl SM buffer by centrifugation under the same conditions and stored at 4 °C until required.

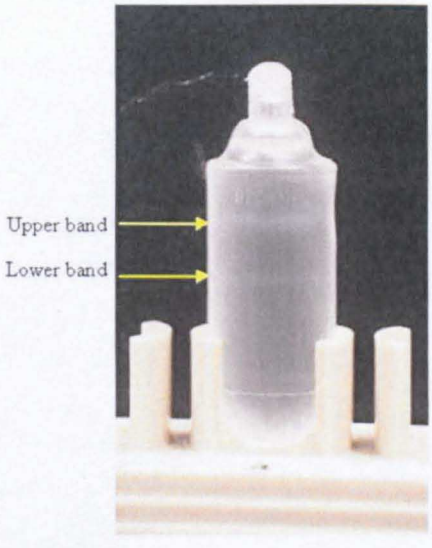


Figure 5.2 CPX Purification

Image shows the two blue bands (upper and lower) resulting from CPX sedimentation using isopycnic caesium chloride gradient centrifugation.

5.2.3 Determination of Detection Threshold of *Campylobacter* Genomic DNA in CPX DNA Preparations

Genomic DNA was prepared from the purified CPX phage (see methods section 2.6.8). To increase the concentration of the purified DNA and overcome problems associated with DNA modification, the samples were amplified using a REPLI-g mini prep kit (Qiagen) as described in the methods section 2.6.9. The quantities of DNA obtained from the phage recovered from the lower and upper bands after CsCl density gradient centrifugation were estimated to be 48.6 and 33.9 ng μl^{-1}, respectively, and following genome amplification using a REPLI-g mini prep kit (Qiagen), were found to be 385.8 and 391.4 ng μl^{-1}, respectively.

To determine the purity of the phage preparation, the level of *Campylobacter* DNA contaminating CPX was determined. *Campylobacter* phage DNA samples were amplified in parallel with a number of *Campylobacter* DNA preparations of known concentration using either *Campylobacter* phage CPt10 primers (CPt10_0091) (Table 5.1) or primers used in previous studies to bind within *Campylobacter* genomic DNA. The primer sequences used for the detection of *Campylobacter* DNA are listed in Table 5.1. Primers AB were used to amplify part of a *Campylobacter*-specific oxidoreductase subunit gene, and therefore specific detection of *C. jejuni* (Nogva *et al.*, 2000). The U primers U515 (Moyer *et al.*, 1998) and U1492 (Menghistu *et al.*, 2011) are universal primers used for the detection of 16s rDNA (Moyer *et al.*, 1998).

Table 5.1 Primer sequences of *Camplylobacter* selected genes

Name	Primer Sequence	Reference
CPt10_ 0091	**F**: 5'- GGC ACT ACT CTA GCC GTT GC -3' **R**: 5'- CAA AAT TTC CAC CAC CTT GC -3'	Timms *et al.*, 2010
AB	**F:** 5'-CTG AAT TTG ATA CCT TAA GTG CAG C-3' **R:** 5'-AGG CAC GCC TAA ACC TAT AGC T-3'	Nogva *et al.*, 2000
U515 U1492	**F:** 5'-GTG YCA GCM GCC GCG GTA A-3' **R:** 5'-GGT TAC CTT GTT ACG ACT T-3'	Moyer *et al.*, 1998 Menghistu *et al.*, 2011

The DNA samples were PCR amplified (see methods section 2.6.10) in TC-312 thermal cycler (Scientific Laboratory Supplies) using the following cycles:

1 cycle	96℃	3 min
35 cycles	96℃ 61℃ 72℃	1 min 1 min 1 min
1 cycle	72℃	7 min

The amplified DNA products were analyzed by electrophoresis on a 0.8 % agarose gel (see methods section 2.6.11) and the image recorded with a ChemiDoc XRS Imager using the Quantity one program (Bio-Rad).

No products were obtained when the pure CPX DNA from either of the two bands from the CsCl density gradient were PCR amplified with *Campylobacter* AB Primers. However, PCR products were obtained when they were PCR amplified using phage primers (CPt10 0091) and with the Universal primers. The detection threshold of *Campylobacter* DNA in both the purified phage

samples was less than 11 pg μl^{-1}, that corresponds to a contamination level <0.003 % (Figure 5.3). This low level of contamination of the phage DNA preparation with *Campylobacter* DNA was considered acceptable for sequence determination.

Following confirmation of the purity of the phage DNA preparation, a 5 µg sample of the DNA prepared from the lower band was used to determine the DNA sequence using Roche 454 pyrosequencing technology performed by DeepSeq in the School of Biomedical Sciences at the University of Nottingham.

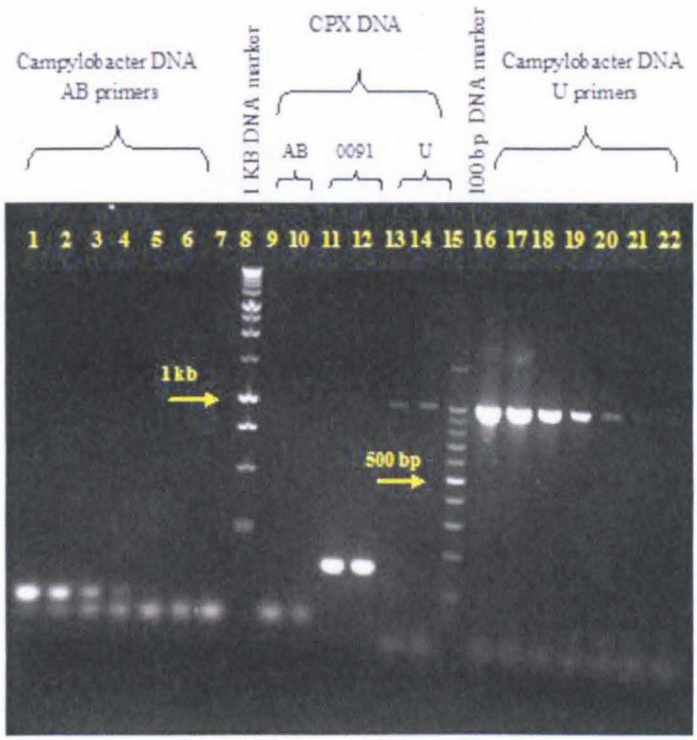


Figure 5.3 Detection of *Campylobacter* DNA threshold level in pure CPX

The image represents a 0.8 % agarose gel of pure CPX and *Campylobacter* DNA of different known concentrations amplified with *Campylobacter* specific and 16 rDNA primers (AB and U respectively) and phage CPt10 0091 primers. Lanes 1-7; *Campylobacter* DNA of known concentration (109.84, 10.984, 1.098 ng μl^{-1}, 110, 11, 1 and 0.1 pg μl^{-1}) amplified with AB primers. Lane 8; 1 kb marker (Promega). Lanes 9 to 14 representsphage CPX DNAs of lower and upper bands amplified with AB primers (lanes 9,10), CPt10 primers (lanes 11, 12) and U primers (lanes 13, 14). Lane 15; 100 bp marker (Promega). Lanes 16-22; *Campylobacter* DNA of known concentration (109.84, 10.984, 1.098 ng μl^{-1}, 110, 11, 1 and 0.1 pg μl^{-1}) amplified with U primers.

5.2.4 The Effect of CPX Phage Infection on *Campylobacter* Growth

To examine the replication of phage CPX on *C. jejuni* PT14, viable counts and phage titres were monitored over a 10 h period using an approximate multiplicity of infection (MOI) of 1, with a starting inoculation of 10^7 CFU ml^{-1} for *Campylobacter* PT14 and 10^7 PFU ml^{-1} for CPX (procedures described in methods section 2.7).

The growth of C. *jejuni* PT14 infected with CPX phage compared with a non-infected culture is shown in Figure 5.4. There was a sharp decrease of 3 $\log_{10}$ CFU ml^{-1} in the *Campylobacter* viable count 1 h after infection with CPX. The cell numbers remained low until the fifth hour, after which there was a steady increase in bacterial cell numbers until the last sampling point. CPX phage numbers increased over the period until the seventh hour when there was a small decrease in the numbers of phage followed by a period when numbers remained relatively stable until the tenth hour.

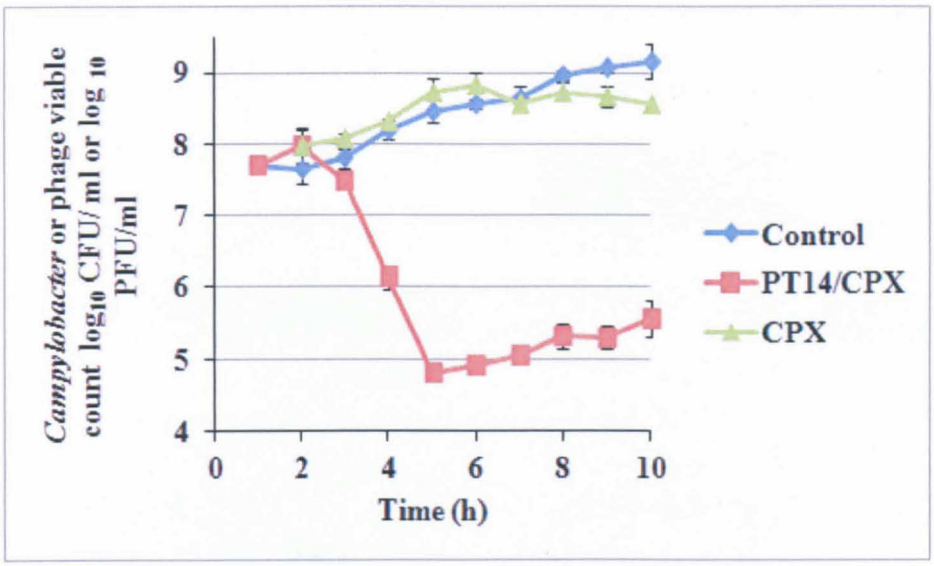


Figure 5.4 Effect of CPX bacteriophage on *C. jejuni* PT14
Graph shows the growth curve for uninfected *Campylobacter* PT14 control, the effect of an MOI 1 of CPX infection of PT14 recorded as the viable bacterial count (CFU ml^{-1}) and the phage titer of CPX (PFU ml^{-1})

5.2.5 CPX DNA Sequences Analysis

The sequence of the amplified CPX DNA was determined and assembled using the DeepSeq facility at the University of Nottingham. Genomic DNA was fragmented to 500 bp using a Covaris S2 sonicator (Covaris Inc., USA) and libraries constructed using a NEBNext DNA Sample Prep Master Mix Set 2 (New England Biolabs Cat. No. E6070S). The libraries were subsequently sequenced using the Roche 454 GS FLX system and assembled using the integral program Newbler (Roche Diagnostics, USA).

The sequenced phage CPX from the sonicated DNA fragments (36,269 reads of average read length 320 bp), resulted in the generation of a single contig of 132,662 bp with minimum redundancy of 44 calls per nucleotide. The complete genome sequence of CPX was submitted to the NCBI Gene bank database where it appears under the accession number JN132397.

5.2.5.1 Sequence Analysis

Statistical analysis of CPX genome was performed using CLC Genomics Workbench 4. The CPX genome was revealed to be a double stranded DNA of 132,662 bp in length, which is composed of 40 % thymine, 34 % adenine, 15 % cytosine and 11 % Guanine. The nucleotide distribution is listed in Table 5.2. The G+C content of CPX is 26.04 % compared to 30.6 % for the host genome of *C. jejuni* (NCTC 11168; Parkhill *et al.*, 2000).

Table 5.2 CPX genome nucleotides distribution

Atom	Count	Frequency
Adenine (A)	45,138	0.340
Cytosine (C)	19,979	0.151
Guanine (G)	14,570	0.110
Thymine (T)	52,975	0.399
C + G	34,549	0.260
A + T	98,113	0.740

5.2.5.2 CPX Annotation

The CPX genome was annotated using the Artemis program (Rutherford *et al.*, 2000), with the help from Dr. Andy Timms (University of Nottingham, School of Biosciences) and was found to contain 149 predicted protein coding sequences (CDS) with the majority of them lying on one DNA strand (126/149). All the open reading frames (ORFs) used ATG as a start codon and used TAA, TGA and TAG as stop codons at the following respective frequencies 67.11 %, 14.09 % and 18.12 %. Most of the CDSs (77/149) were identified as hypothetical phage proteins with no assigned function. However, many were similar to *Campylobacter* phage NCTC 12673 (Kropinski *et al.*, 2011), and a few similar to CPt10 (Timms *et al.*, 2010).

The genome was found to contain five transfer RNA (tRNA) genes with anticodons for the amino acids; tyrosine (Tyr, GTA), arginine (Arg, TCT), aspargine (Asn, GTT), leucine (Leu, TAA) and methionine (Met, CAT).

A search for insertion sequences was performed using IS Finder (Siguier *et al.*, 2006). This phage does not possess intact insertion sequences, although there are some very short sequences with similarities to known insertion sequences from different microorganisms that CPX would be unlikely to infect. However, a short sequence match to IS607 was observed, which originates from *Campylobacter fetus* subsp *venerealis*. A BLAST search showed that similarity to a group of unrelated genes that have homing endonuclease-like function (Hef) from *Campylobacter* phage NCTC12673 was found in seven CDS's. In

addition to that, the CPX genome exhibited two repeat regions with a number of repeat units within.

Some of the identified genes were similar to structural and enzymatic genes from T4 phage (Table 5.3). The rest of the genome contained genes identified to have possible functions such as packaging enzymes, reductases, decarboxylases, helicases, repair and recombination proteins, methylases, kinases, peptidases, topoisomerases or dehydrogenases.

Table 5.3 Putative structural and enzymatic genes similar to T4 phage encoded by bacteriophage CPX

Location	Gene	Possible function
C* 1454...2191	gp2	DNA end protector protein
C* 7987...9897	gp18	Tail sheath protein
C* 11373...12695	gp30	DNA ligase
C* 19079...20476	gp23	Major capsid protein
C* 21264...21893	gp21	Prohead core scaffold and protease
C* 22058...23764	gp20	Portal vertex protein
24513...25244	gp13	Neck protein
26852...27583	gp19	Tail tube protein III
C* 843284...45947	gp43	DNA polymerase
C* 48558...49502	gp61	DNA primase subunit
50491...50946	gp3	Tail completion and sheath stabilizer protein
C* 56056...57660	gp46	Recombination endonuclease
C* 58123...59118	gp47	Recombination endonuclease
C* 59106...59975	gp55	Sigma factor for T4 late transcription
C* 60067...61029	gp23	Major head protein
61039...61686	gp14	Head completion protein
63558...64589	gp15	Tail sheath stabilizer and completion protein
C* 77454...77897	gp4	Head completion protein
C* 90232...9116	gp44	Sliding clamp loader subunit

* C used to indicate that this gene is located on the complementary strand

The annotated genome showed some significant differences in 11 regions over the whole genome compared to available *Campylobacter* phage genome sequences. There was a need to confirm DNA sequences to check that in the gaps between the identified CDS regions there were no sequencing errors, and to check that in regions where two or more smaller CDS regions were identified compared to existing group III phage DNA sequences that there were no unintended frame shifts. To confirm the DNA sequences, PCR was carried out to amplify the DNA from these regions as performed earlier (see sections 2.6.10 and 5.2.3) using primers designed for these regions (Table 5.4). The PCR products were analysed by gel electrophoresis (Figure 5.5), purified (see section 2.6.13) and the products sequenced and analyzed using BioEdit. The newly obtained sequences were identical to the sequenced genome. Thus no changes were made to that determined from the 454 contig assembly. The full annotation of CPX is listed in Appendix 3.

Table 5.4 Primer sequences of CPX genome irregular regions

Region number	Position	Primer Sequence
1	>2034..2979	F: 5'-TTGCCCTGATACCATTGTTG-3' R: 5'-CCTTGTGACATATCAGAATAATCACC-3'
2	>40602..42087	F: 5'-CCTAAAGATCAAACTGCACTTATTAAA-3' R: 5'-ACGCTTGCTCGTTATCATCA-3'
3	>50160..51168	F: 5'-CCTAAAGATCAAACTGCACTTATTAAA-3' R: 5'-GAGCAAAACACAGTTGTATCAGC-3'
4	>53902..55371	F: 5'-GCAAAATGGGGTGTTGGTAG-3' R: 5'-TGATTGCTCTTGTGTTCCATCT-3'
5	>67891..69594	F: 5'-AGGCTTACCCCCAAAAGAAA-3' R: 5'-AAAAACTTTTCCTGTTGATTTTCA-3'
6	>73966..75021	F: 5'-AGACGGTAAAATTGATTATGAAAAC-3' R: 5'-AATAGCCTTAAAGTTGGCTTAAAA-3'
7	>76525..77940	F: 5'-GCTGGTGTTCTTAAATGGGCTA-3' R: 5'-TTGATTCTTTTAACTTCTCTTGTTGC-3'
8	>85576..87972	F: 5'-CACATTTTTGAACATTCATGG-3' R: 5'-CCATTGTACCAACACAACTACCA-3'
9	>91393..92808	F: 5'-AATGGGCTCTGCTTGTTACG-3' R: 5'-CACACAATAAAATGAAGTCACCTCT-3'
10	>127105..128079	F: 5'-GGCTCAAAGAACTTAATCAAACAA-3' R: 5'-GCTTCAGATGTAAGAACTTTTAATGG-3'
11	>128078..128908	F: 5'-TCAAATACATTTACAGGTGATCAAA-3' R: TCCGCAGATTCGTATGTATTTT-3'

Figure 5.5 CPX pure PCR products

To confirm the CPX genome sequence in regions showing irregularities. DNA amplification was carried out using primers designed for them. PCR products were purified and examined using 0.8 % agarose gel electrophoresis. Lanes representing the 11 irregular regions are as follows;

Lane 1; 1 kb marker (Promega), Lanes 2-12; purified PCR products of the irregular regions 1 to 11

5.2.6 Mass Spectrometry (MS) of CPX Phage

The purified CPX phage particles were TCA precipitated (see methods section 2.12) before identification of the capsid protein sequences using Mass Spectrometry (Q-TOF MS/MS described in section 2.9).

The protein profile of the phage CPX particles is shown in Figure 5.6. The major band was excised from the gel and cleaved with trypsin before identification of the peptides based on the mass prediction of the translated CPX DNA sequence and *de novo* sequencing using mass spectrometry (Q-TOF

MS/MS). The *de novo* peptide sequences obtained are listed in Table 5.5. A BLASTP search of these peptide sequences within the NCBInr database showed that these peptide sequences produced significant matches with a hypothetical protein from *Campylobacter* phage CP81 or gp23 major capsid protein from *Campylobacter* phage NCTC12673. An example of the search output for one peptide (867) is shown in Figure 5.7. The sequences from phage CP81 and NCTC12673 were aligned together with the identified peptides to show their relative positions (Figure 5.8).

Figure 5.6 Denaturing protein gel electrophoresis of TCA precipated CPX
To identify the proteins in CPX particles , the phage was treated with TCA and examined using 10-20 % Novex Tricine gel. Lanes representing the treated samples are as follows;
Lanes 1-2; Purified CPX phage treated with TCA, lane 3; See Blue Plus Pre-stained standard (Invitrogen)

Table 5.5 *De novo* Peptide Sequences

Peptide 866	NDFNYTGTPLEVSFK	it is not possible to determine the order of the first 2 residues- it could be ND or DN
Peptide 700	QAGTDDVVNVLLR	
Peptide 867	LESNSTGSVAIGDEIDK	it is not possible to determine the order of the first 2 residues- it could be LE or EL
Peptide 878	ELADILSAEVALEIDR	
Peptide 1021	VATVCTDFDVNSADGR	partial sequence obtained for this peptide

Peptide 867 LESNSTGSVAIGDEIDK

```
emb|CBZ42322.1|  hypothetical protein [Campylobacter phage CP81]
Length=465
 Score = 54.1 bits (120),  Expect = 2e-08
 Identities = 17/17 (100%), Positives = 17/17 (100%), Gaps = 0/17 (0%)

Query  1    LESNSTGSVAIGDEIDK  17
            LESNSTGSVAIGDEIDK
Sbjct  167  LESNSTGSVAIGDEIDK  183


ref|YP_004421627.1|  gp23 major capsid protein [Campylobacter phage NCTC12673]
gb|AEA86419.1|  gp23 major capsid protein [Campylobacter phage NCTC12673]
Length=465
GENE ID: 10896891 NCTC12673_gp074 | gp23 major capsid protein
[Campylobacter phage NCTC12673]

 Score = 54.1 bits (120),  Expect = 2e-08
 Identities = 17/17 (100%), Positives = 17/17 (100%), Gaps = 0/17 (0%)

Query  1    LESNSTGSVAIGDEIDK  17
            LESNSTGSVAIGDEIDK
Sbjct  167  LESNSTGSVAIGDEIDK  183
```

Figure 5.7 NCBInr BLASTP Output

Figure 5.8 Sequence alignment of identified peptides against phage CP81 and NCTC12673 GP23 proteins

5.2.7 Comparison of CPX Genome to Other *Campylobacter* Bacteriophage Genomes

CPX was compared to other available sequenced *Campylobacter* phages (Table 5.6), which included; CP220, CPt10 (Timms *et al.*, 2010), CP81 (Hammerl *et al.*, 2011), NCTC 12673 (Kropinski *et al.*, 2011) and CP21 (Hammerl *et al.*, 2012). CPX belongs to the same class (III) as CP81 and NCTC 12673, whereas CP220, CPt10 and CP21 belong to class II. CPX has fewer predicted CDS genes compared to the published phage sequences. Two DNA repeat regions were identified in CPX compared with eight in NCTC 12673, 45 in CPt10, 82 in CP220, four in CP21 and none in CP81. CPX and CP81 contain five tRNAs. However, NCTC 12673 has three tRNAs, CP220 and CPt10 have two each and CP21 has one. All the phages are AT rich with similar GC content. Phage vB_CcoM-IBB_35 (Carvalho *et al.*, 2012) is another available phage that belongs to group II but was not included in the analysis as it consists of five contigs which are not assembled.

A comparison of CPX with other phages performed using Mauve genome alignment program (Darling *et al.*, 2010) showed the greatest similarity of CPX phage to phage CP81 followed by phage NCTC 12673 (Figure 5.9), which like CPX are class III phages. CPX phage is different from CP220, CPt10 (Figure 5.10) and CP21(Figure 2.11) phages that are class II.

Table 5.6 Comparison of basic parameters from published phage genomes

Phage	Genome size (bp)	Genome % G+C content	Number of CDS
Class II phages			
CP220	177 500	27.4	194
CPt10	175 700	27.3	201
vB_CcoM-IBB_35	172 065	27.0	210
CP21	182 833	27.2	259
Class III phages			
CPX	132 662	26.04	149
CP81	132 500	26.1	188
NCTC 12673	135 000	26.2	172

Figure 5.9 Comparison of CPX to CP81 and NCTC 12673

The graphs represents genome sequences comparison between **A**; CPX (TOP) and CP81 (Bottom), **B**; CPX (TOP) and NCTC 12673 (Bottom). In each comparison block of the same colour between two similar regions within the genomes are connected by lines. The colours within these blocks show the strength of homology whereas the white regions are not aligned or might be specific to particular phage. Blocks above centre lines lies in the same orientation and the blocks below it lies in the opposite orientation.

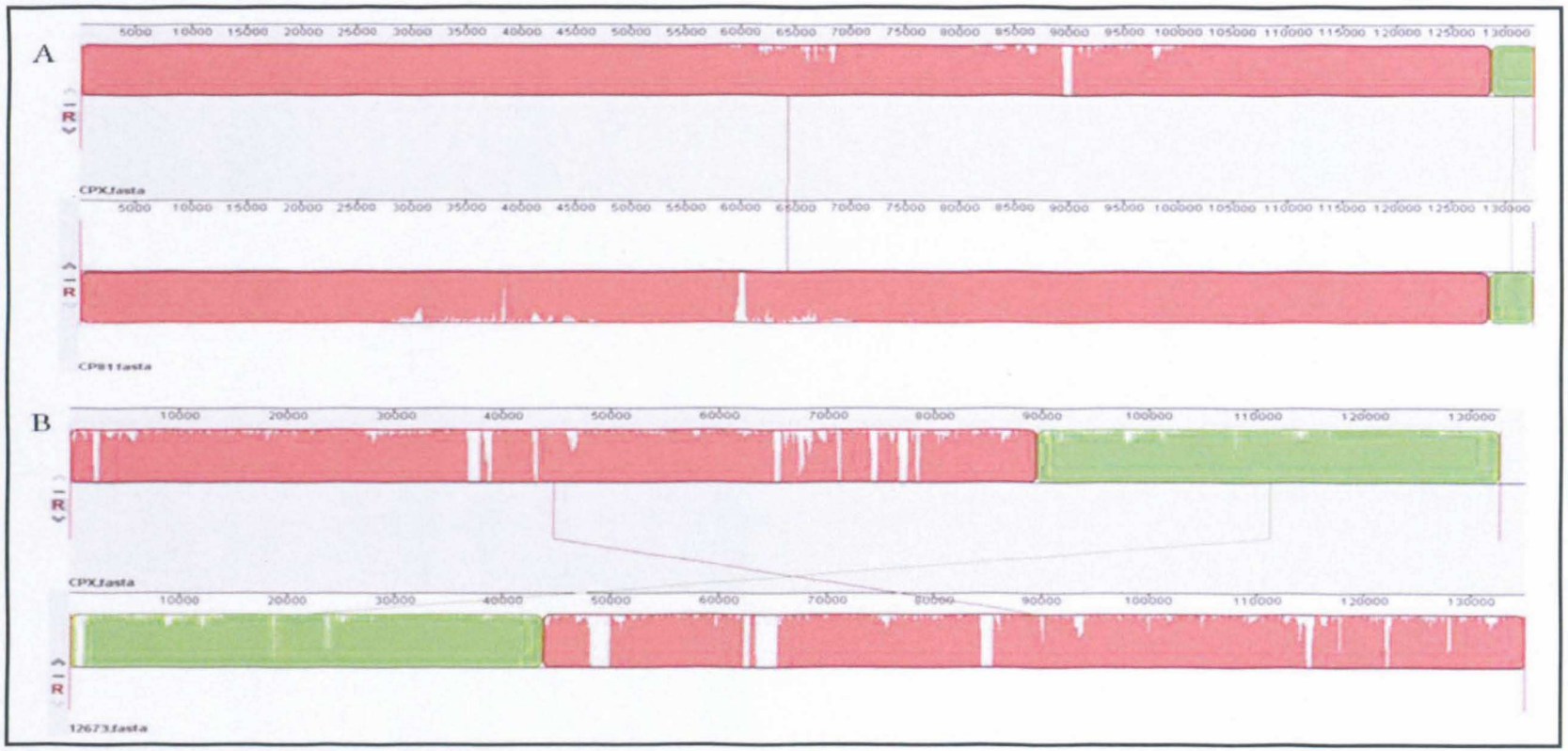


Figure 5.10 Comparison of CPX to CP220 and CPt10

The graphs represents genome sequences comparison between **A**; CPX (TOP) and CP220 (Bottom), **B**; CPX (TOP) and CPt10 (Bottom). In each comparison block of the same colour between two similar regions within the genomes are connected by lines. The colours within these blocks show the strength of homology whereas the white regions are not aligned or might be specific to particular phage. Blocks above centre lines lies in the same orientation and the blocks below it lies in the opposite orientation.

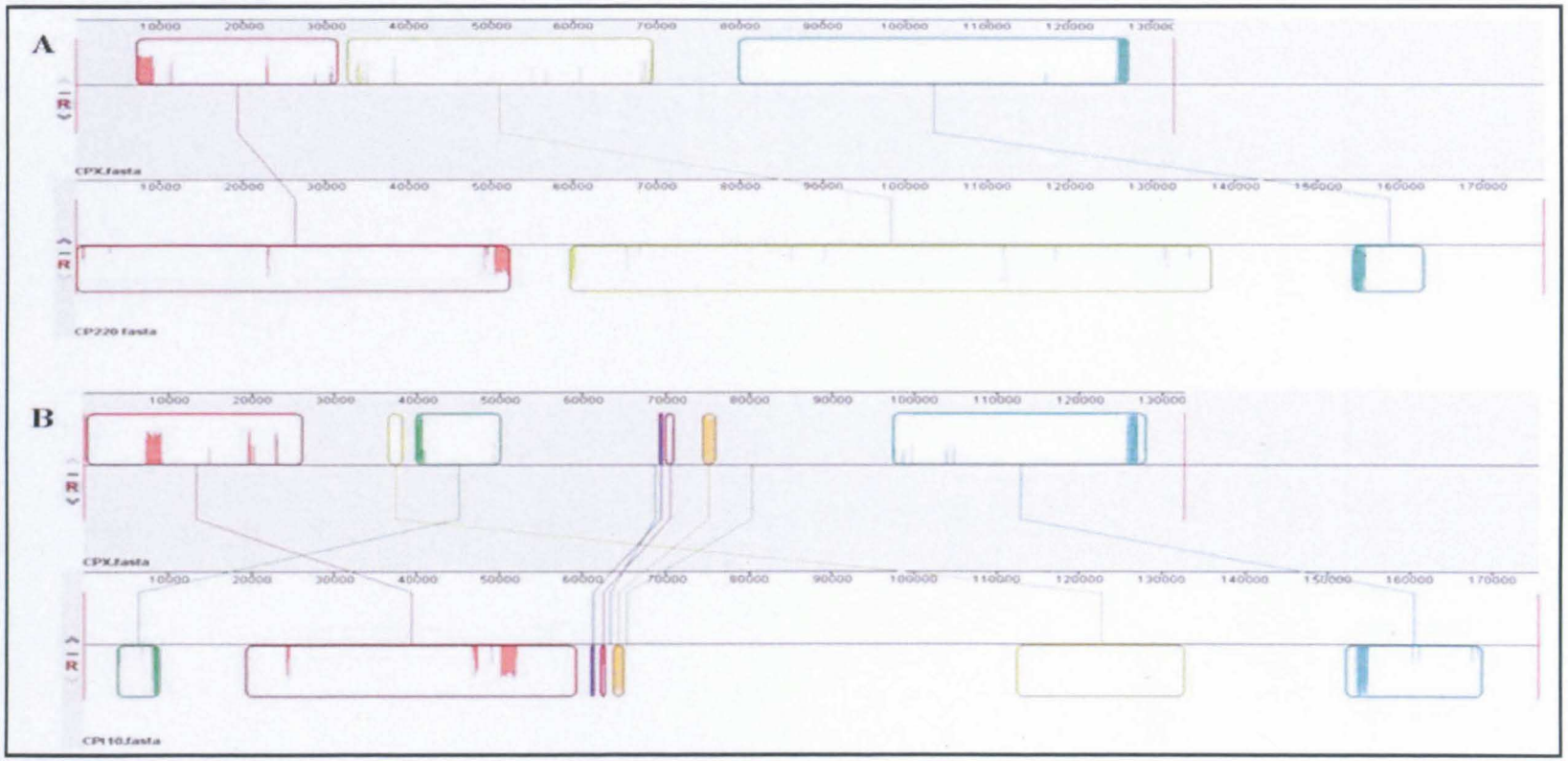
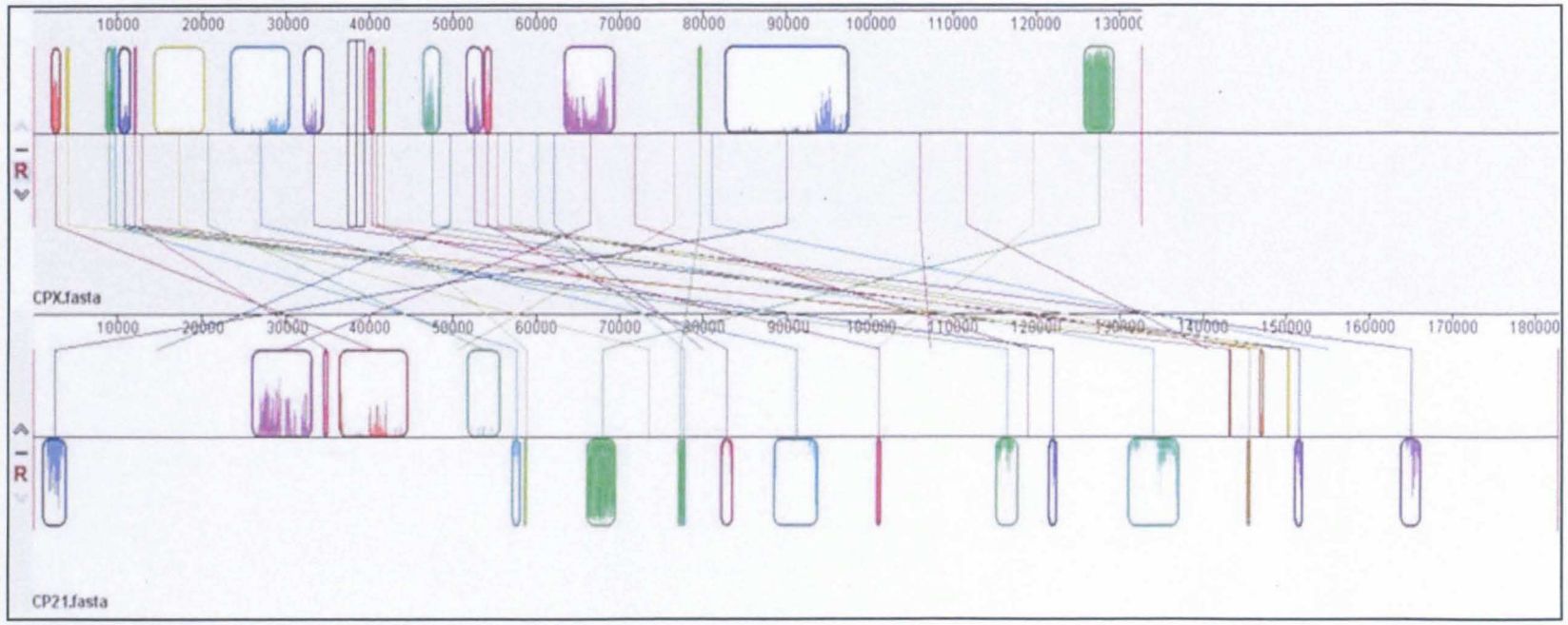


Figure 5.11 Comparison of CPX to CP21

The graph represents genome sequences comparison between CPX (TOP) and CP21 (Bottom). The blocks of the same colour between two similar regions within the genomes are connected by lines. The colours within these blocks show the strength of homology whereas the white regions are not aligned or might be specific to particular phage. Blocks above centre lines lies in the same orientation and the blocks below it lies in the opposite orientation.



5.3 Discussion

The main purpose of the work described in this chapter was to obtain and study the sequence of CPX to determine if it had any relationship to CP220 in order to investigate if there was any significance in their co-isolation, and to generate more sequence data to increase our knowledge of *Campylobacter* phage.

As no studies of this phage had been previously carried out, the growth characteristics of CPX phage were studied and it was found to exhibit a typical phage growth curve when infecting its host bacteria, *C. jejuni* PT14 (Loc Carrillo *et al.*, 2005). Cairns *et al.* (2009) found that the increase in phage numbers could be seen only after *Campylobacter* had reached 7 $\log_{10}$ CFU ml^{-1} (the phage proliferation threshold). Based on this finding and to study the effect of CPX on *C. jejuni* PT14 the initial concentration of 7 $\log_{10}$ CFU ml^{-1} was chosen with a multiplicity of infection (MOI) of 1.

Campylobacter phages are divided into three groups based on their genome size (Sails *et al.*, 1998). The first *Campylobacter* phages to be sequenced, CP220 and CPt10, belonged to group II (Timms *et al.*, 2010). Sequences of phages that belong to group III have only recently been reported, and include CP81 (Hammerl *et al.*, 2011), NCTC 12673 (Kropinski *et al.*, 2011) and CPX described here. There is no sequenced phage that belongs to group I. The reason why so few *Campylobacter* phage sequences have been available until recently is due to the technical difficulties of sequencing genomes consisting of non-standard bases when sequencing relied on being able to clone fragments of phage DNA into *E. coli* vectors. Recent developments in sequencing, including

pyrosequencing technology, which involves sequencing by synthesis in the Roche 454 GS FLX system that was performed by DeepSeq at the University of Nottingham, enabled the sequence of CPX phage to be determined. Before sequencing could commence it was important to produce good quality DNA, free from *Campylobacter* DNA contamination. Caesium chloride density gradient centrifugation was employed to separate the phage particles from lysed cell debris and methods were developed employing PCR primers, to check that the residual *Campylobacter* DNA content was at a level low enough not to affect the elucidation of the phage sequence. These methods will be useful for determination and quality control of other phage sequences in the future.

When the sequence became available it was analyzed in terms of its relationship to phages from other bacteria and specifically to *Campylobacte*r phage CP220 that was its co-isolate. There were no regions of extended nucleotide homology between the CP220 and CPX genomes, implying they are not derivatives of each other. The relationship between these phages, if any, could have a functional rather than evolutionary basis. Genomes of different phages have been shown to possess different numbers of tRNAs. The CPX genome exhibited five tRNAs similar to phage CP81 (Hammerl *et al.*, 2011), whereas CP220 and CPt10 (Timms *et al.*, 2010) have two tRNAs and NCTC 12673 has three tRNAs (Kropinski *et al.*, 2011). For comparison the prototype *E. coli* phage T4 genome contains eight tRNAs (Miller *et al.*, 2003).

Like phages CP220, CPt10 (Timms *et al.*, 2010), CP81 (Hammerl *et al.*, 2011), NCTC 12673 (Kropinski *et al.*, 2011) and indeed T4 (Miller *et al.*, 2003), CPX is A+T rich, which might be due to the preference for A or U in the third position of the codons (Timms *et al.*, 2010).

In relation to T4–like phages that have genomic size ranges between 164 and 255 kb, CP220 (177.5 kb) and CPt10 (175.7 kb) can be classified as T4 related phages, whereas CP81 (132.5 KB) NCTC 12673 (135.0 KB) and CPX (132.6 Kb) are not so similar since their molecular size is less than the smallest characterized T4 genome, even though they have some genes in common with T4 related phages (Hammerl *et al.*, 2011).

A large proportion of the putative CDSs (78/149) showed no significant matches to available sequences in data bases. This is similar to CP220 and CPt10 where 85 out of 194 and 95 out of 201 open reading frames did not show any similarities (Timms *et al.*, 2010).

Homing endonuclease encoding genes (*Hef*) have been identified in *Campylobacter* phages (Timms *et al.*, 2010). Of the group III phage, CPX has seven Hefs compared with NCTC12673 that has twelve (Kropinski *et al.*, 2011), whereas CP81 has eight (Hammerl *et al.*, 2011). Homing endonucleases are essentially selfish genetic elements that cleave chromosomes that do not contain them, and then get copied across to the broken chromosome as a byproduct of the repair process. In this way they can disseminate themselves and can instigate horizontal gene transfer (Belfort and Roberts 1997).

One reason why the genomes of CP220 and CPt10 are larger than those of the group III phages is probably because they include proteins that are not encoded in CP81 (Hammerl *et al.*, 2011), NCTC12673 (Kropinski *et al.*, 2011) or CPX. These include transposases, metabolic enzymes and notably S-adenosylmethionine (SAM) domain proteins.

The reason for the co-existence of CPX with CP220 remains unclear. The two phages did not appear to share significant sequence homology indicative of exchange of genetic material due to their close environmental proximity, although like many phages they do share genes with similar functions. It is likely that both phages have evolved separately to successfully exploit the same host and are able co-exist without competing with each other for resources, making it difficult to separate them using current phage isolation techniques. The efficiency of CPX in reducing *Camplylobacter* numbers *in vitro* together with the novel genome sequence that distinguishes it from CP220, makes this phage a unique and a promising agent for phage therapy, where it might be used in a cocktail of several phages to increase the possibility of reducing the viability of a wide range of *Campylobacter* hosts.

CHAPTER SIX

GENE CLONING AND PROTEIN EXPRESSION FROM BACTERIOPHAGE CP220

6.1 Introduction

The bacteriophage CP220 genome sequence (Timms *et al.*, 2010) provided a resource to search databases for putative protein products that catalyse reactions with bacterial cell wall components with the potential to lyse bacterial cells. Two reading frames were identified encoded by CPT 0075 and CPT 120. CPT 0075 encodes a PAAR (perfect amino acid repeat) containing protein with 48 % identity to phospholipase A1 from *Agrobacterium vitis* S4 and *Dickeya dadantii*. This PAAR motif can be found either in a family of membrane proteins in pairs or in another family of hypothetical proteins as triplets of tandem repeats comprising the entire length. CPT 120 showed 42 % identity to a murein-degrading enzyme from *Filifactor alocis* ATCC 35896 (annotated as soluble lytic transglycosylase or murein hydrolase B). This enzyme has bacteriolytic activity as it catalyses the cleavage of glucosidic bonds between N-acetyl muramic acid and N-acetylglucosamine residues in cell wall peptidoglycans.

Gene CPT 0075 is 293 bp in size and encodes a protein with a predicted molecular weight of 9.5 kDa, whereas gene CPT 120 is 674 bp in size and encodes a protein with a predicted molecular weight of 26.3 kDa. In order to assess if these predicted protein products have lytic functions, the corresponding reading frames need to be expressed and the resulting proteins analysed.

To produce sufficient amounts of such proteins to enable their functions to be characterised, they must be expressed in a suitable host using an expression vector that is host compatible, contains a tightly controlled inducible promoter, a sequence that terminates the transcription and a ribosomal binding site. Different expression systems are available each with advantages and disadvantages. The widely used *E. coli* expression system pET was selected for this study. Transcription is directed from a T7 promoter present on the expression vector that is bound by T7 RNA polymerase expressed from the *E. coli* host chromosome under control of the *lac* promoter. The design of this system is such that the only target of the T7 polymerase is the gene to be expressed, and that any T7 polymerase expressed before induction is inhibited by the presence of T7 lysozyme (pLysE or pLysS) located and expressed from a separate plasmid. In this way the pET system enables the control of both the timing and the quantity of the protein to be expressed.

To accomplish the aim of expressing the target genes from CP220, primers were designed to amplify the CPT 0075 and CPT 120 reading frames with the addition of specific restriction sites to be incorporated at the ends of the PCR products (*Nde*I/*Bam*HI) to allow their sub cloning into the expression vector. The CP220 genes were PCR amplified before cloning and sequencing from the plasmid pCR2.1-TOPO using the non-expression host *E. coli* Top10 chemically competent cells. Once characterised, the DNA fragments were subcloned from pCR2.1-TOPO into the pET expression plasmids before transforming these in to a number of *E. coli* expression hosts including BL21 (DE3) pLysS, BL21 (DE3) pLysE and Rosetta 2 (DE3) pLysS.

6.2 Results

6.2.1 Cloning of CP220 Potential Lysin Genes

The reading frames of CP220 potential lysins CPT 0075 and CPT 120 were PCR amplified as described in methods section 2.6.9 and cloned into the plasmid vector pCR2.1-TOPO and transformed into *E. coli* TOP 10 chemically competent cells and plated on LB agar containing ampicillin (2.4.1) and the chromogenic substrate X-gal (see methods sections 2.8.2 and 2.8.3). Five white coloured ampicillin resistant colonies were selected from each of the transformation plates as prospective TOP 10 *E. coli* bearing plasmid pCR2.1-TOPO in which the target PCR amplified DNAs were inserted within the cloning site and thereby disrupting the expression of *lac*Z. From these plasmid DNAs were prepared and digested using the restriction endonuclease *Eco*RI, sites for which flank the cloning site of plasmid pCR2.1-TOPO (see methods sections 2.8.4 and 2.8.5). The results of agarose gel electrophoresis of the digested plasmid DNAs are shown in Figures 6.1 and 6.2. From these, 4/5 clones contained the DNA insert corresponding to CPT 0075 (Figure 6.1) and 5/5 the DNA inserts for CPT 120 (Figure 6.2). The DNA sequences of two positive clones of each gene were determined through Eurofins MWG Operon and the sequences obtained analyzed using BioEdit. The sequences of CPT 0075 and CPT 120 cloned in pCR 2.1-TOPO are listed in Appendix 4.

Figure 6.1 Plasmids digests of CP220 CPT 0075 clones in pCR2.1-TOPO

In order to check the presence of the correct insert in pCR2.1-TOPO, plasmids were prepared, digested with *Eco*RI and analyzed by 0.8 % agarose gel electrophoresis. Lanes representing these plasmids are as follows:

Lanes 1-2; colony 1, lanes 3-4; colony 2, lanes 5-6; colony 3, lanes 7-8; colony 4, lanes 9-10; colony 5, lane 11; 1 kb marker

* First lane of each sample was undigested plasmid; the second lane was digested with *Eco*RI

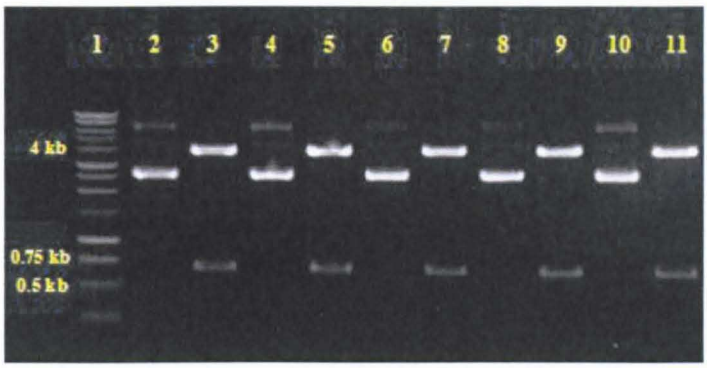


Figure 6.2 Plasmids digests of CP220 CPT 120 clones in pCR2.1-TOPO

In order to check the presence of the correct insert in pCR2.1-TOPO, plasmids were prepared, digested with *Eco*RI and analyzed by 0.8 % agarose gel electrophoresis. Lanes representing these plasmids are as follows:

Lane 1; 1 kb marker, lanes 2-3; colony 1, lanes 4-5; colony 2, lanes 6-7; colony 3, lanes 8-9; colony 4, lanes 10-11; colony 5

* First lane of each sample was undigested plasmid; the second lane was digested with *Eco*RI

6.2.2 Cloning of CPT 0075 and CPT 120 Genes in pET 3a

The pCR2.1-TOPO plasmid vectors carrying CP220 genes CPT 0075 and CPT 120 were subcloned into pET 3a. These plasmids were digested to release the inserts using *Nde*I and *Bam*HI to get the right terminal sequences for their ligation in to similarly treated expression plasmid pET 3a (see methods section 2.8.6.1). The digested plasmids (Figure 6.3) were run on 0.8 % low melting point agarose gels and purified using a gel extraction kit (see methods sections 2.6.10 and 2.6.11). The DNA inserts were ligated into the vector using T4 DNA ligase at a molar ratio of 3:1 insert:vector (see methods section 2.8.6.4). The molar ratio was calculated using the following formula: [(100 ng vector * kb insert) / kb vector) * (3/1)] = A ng insert

The reaction mixture used was:

Components	CPT 0075		CPT 120	
	Concentration	Volume	Concentration	Volume
Vector (pET 3a)	50 ng	1.9 µl	50 ng	1.9 µl
Insert	9.8 ng	5.9 µl	22 ng	2.8 µl
Ligase buffer (10 X) (Promega)	1 X	1.0 µl	1 X	1.0 µl
T4 DNA ligase (Promega; 10 U µl^{-1})	1 U	1.0 µl	1 U	1.0 µl
Nuclease free water (Up to 10.0 µl)	-	0.2 µl	-	3.3 µl

After incubation, the ligation mixture was transformed into *E. coli* TOP10 chemically competent cells (prepared as described in section 2.8.3). The plasmids were prepared from a number of clones to check for the insert presence in pET 3a. Figures 6.4 and 6.5 show respectively 4/8 positive clones for CPT 0075 (Figure 6.4) and 4/6 positive clones for CPT 120 (Figure 6.5). The positive plasmids from two of the clones were sequenced by Eurofins MWG Operon and analyzed using BioEdit. The sequences of CPT 0075 and CPT 120 in pET 3a are listed in Appendix 5.

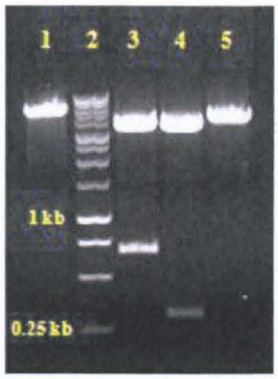


Figure 6.3 Vectors and inserts digested with *Nde*I and *Bam*HI

Insert DNAs and plasmid vectors (pET) digested with *Nde*I and *Bam*HI and electrophoresed on 0.8 % low melting point agarose gel. Lanes representing these fragments are as follows:

Lane 1; pET 11a, lane 2; 1 kb marker, lane 3; CP220 CPT 120, lane 4; CP220 CPT 0075, lane 5; pET 3a

Figure 6.4 Restriction digest of CP220 CPT 0075 plasmid clones in pET 3a

In order to check the presence of the correct insert in pET 3a, plasmids were prepared, digested with *Nde*I and *Bam*HI and analyzed by 0.8 % agarose gel electrophoresis. Lanes representing these plasmids are as follows:

Lane 1; 1 kb marker, lanes 2-3; colony 1, lanes 4-5; colony 2, lanes 6-7; colony 3, lanes 8-9; colony 4, lanes 10-11; colony 5, lanes 12-13; colony 6, lanes 14-15; colony 7, lanes 16-17; colony 8

* First lane of each sample was undigested plasmid DNA; the second lane was digested with *Nde*I and *Bam*HI

Figure 6.5 Restriction digest of CP220 CPT 120 plasmid clones in pET 3a

In oder to check the presence of the correct insert in pET 3a, plasmids were prepared, digested with *Nde*I and *Bam*HI and analyzed by 0.8 % agarose gel electrophoresis. Lanes representing these plasmids are as follow:

Lane 1; 1 kb marker, lanes 2-3; colony 1, lanes 4-5; colony 2, lanes 6-7; colony 3, lanes 8-9; colony 4, lanes 10-11; colony 5, lanes 12-13; colony 6, lanes 14-15; colony 7, lanes 16-17; colony 8

* First lane of each sample was undigested plasmid DNA; the second lane was digested with *Nde*I and *Bam*HI

6.2.3 Expression of CPT 0075 and CPT 120 Proteins

Expression plasmid DNAs carrying the genes encoding CPT 0075 and CPT 120 were transformed into chemically competent *E. coli* expression host strains detailed in Table 6.1 to enable protein expression. The transformation mixtures were plated on LB agar plates containing ampicillin (see section 2.4.1) and chloroamphenicol (see section 2.4.2) to select respectively for the pET and pLys plasmids. The plates were incubated at 37 °C for 24 h. The resultant colonies were used for protein expression studies. Cultures of these transformants were induced with 0.1 mM IPTG (2.3.8) for 4 h and total cell fractions were collected and examined as described in methods section 2.8.7.

Table 6.1 *E. coli* hosts

Non Expression Host	**Genotype**	**Reason for Selection**
TOP10 chemically competent cells	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Standard host for transformation
Expression Hosts	**Genotype**	**Reason for Selection**
BL21 (DE3) pLysS	F– *ompT* *hsd*SB(rB–, mB–) *gal dcm* (DE3) pLysS (CamR)	Carries an IPTG-inducible T7 RNA polymerase in a genetic background deficient in both *lon* and *ompT* proteases. The pLys S plasmid carries the gene encoding T7 lysozyme, which can lower any background expression of the target genes without interfering with the expression level following IPTG induction.
BL21 (DE3) pLysE	F– *ompT* *hsd*SB(rB–, mB–) *gal dcm* (DE3) pLysE (CamR)	As above but provides tighter control of T7 RNA polymerase
Rosetta 2 (DE3) pLysS	F⁻ *ompT* *hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	As above and is able to supply tRNAs for rare codons.

The pET 3a expression plasmids were transformed into the *E. coli* expression hosts BL21 (DE3) pLysS and BL21 (DE3) pLysE. However, no protein expression was detected in either BL21 (DE3) pLysS or BL21 (DE3) pLysE by denaturing polyacrylamide gel electrophoresis (not shown) for clones carrying either CPT 0075 or CPT 120. A different *E. coli* expression host, Rosetta 2 (DE3) pLysS was then chosen, as this host could supply tRNAs for rare codons. Whilst CPT 0075 did not yield any protein of the expected molecular mass 9.5 kDa, CPT 120 was expressed successfully producing a protein of 26 kDa (Figure 6.6) after induction with 0.1 mM IPTG.

Tricine (10-20 %) gels were run (see methods section 2.8.10) and stained for protein with colloidal Coomassie Blue. The additional protein band identified from the cultures expressing CPT 120 was excised before tryptic digestion and protein sequencing by mass spectrometry (Q-TOF) to confirm that it is the target (see methods section 2.9) .

Figure 6.6 Protein expression of CP220 CPT 0075 and 120

To examine the protein expression in pET 3a/Rosetta 2 (DE3) pLysS, total cell proteins from *E. coli* cultures harvested pre- and post-induction with 0.1 mM IPTG carrying either the CP220 target genes or an empty vector control, were electrophoresed on 10-20 % Tricine gels. Lanes representing these samples are as follows:

Lanes 1-2; CP220 CPT 120, lanes 3-4; CP220 CPT 0075 sample 1, lanes 5-6; CP220 CPT 0075 sample 2, lanes 7-8; negative control (pET 3a in Rosetta cells), lane 9; pre-stained See blue plus 2 protein marker

* First lane of each sample is pre induction and the second lane is after 4 hours of induction with IPTG

6.2.4 CPT 0075 Cloning in pET 11a

CP220 gene CPT 0075 was not expressed in any of the three *E. coli* expression hosts tried carrying the T7 polymerase. T7 RNA polymerase can be expressed even in the absence of IPTG. If the target gene is toxic to *E. coli*, then its expression will be prevented. For this reason, pET 11a was selected as an alternative vector where the target gene can be cloned using the same cloning sites (*Nde*I and *Bam*HI). This vector contains a T7 *lac* promoter, where the presence of *lac* operator is useful in blocking transcription of genes by preventing uninduced T7 RNA polymerase expression. The plasmid carrying the CPT 0075 gene and pET 11a vector were digested with *Nde*I and *Bam*HI (Figure 6.3) and ligated as described when pET 3a was the choice of vector (see sections 6.2.2 and 6.2.3) with the exception of the vector:insert ratio. The amount of vector and insert to be ligated was calculated and the following mixture was used:

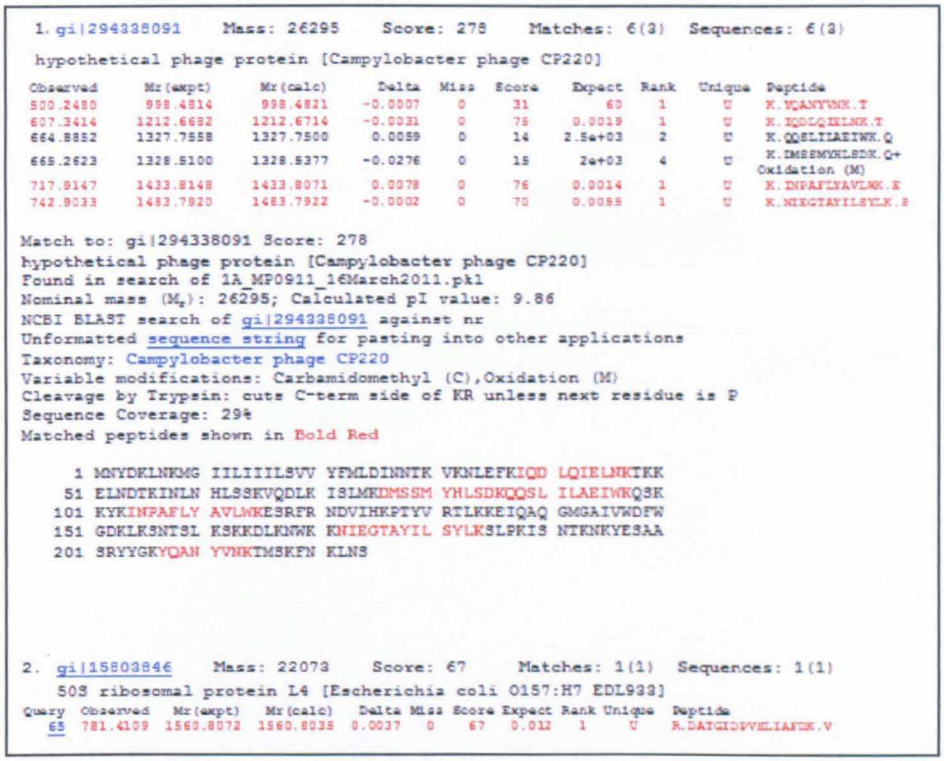
Components	Concentration	Volume
Vector (pET 11a)	50 ng	2.3 µl
Insert (CPT 0075)	8 ng	3.0 µl
Ligase buffer (10 X) (Promega)	1 X	1.0 µl
T4 DNA ligase (Promega; 10 U µl^{-1})	1 U	1.0 µl
Nuclease free water (Up to 10.0 µl)	-	2.7 µl

The CPT 0075 gene was successfully cloned into pET 11a. The *E. coli* cultures resulting from the transformation of the plasmid into the expression hosts BL21 (DE3) pLysE and Rosetta 2 DE3 pLysS were examined for protein expression by denaturing polyacrylamide electrophoresis but did not yield any recombinant protein as determined by visualization on 10-20 % Novex Tricine gels (described in methods section 2.8.10).

6.2.5 Mass Spectrometry

Cellular protein of *E. coli* expressing CPT 120 from pET 3a in Rosetta 2(DE3) pLysS was loaded on a 10-20 % Novex Tricine gel and stained with colloidal coomassie blue (Figure 6.6) before excising the protein bands for identification of the tryptic fragments by Mass Spectrometry (described in methods section 2.9).

Tryptic digestion of the protein band recovered for CPT 120 expressed in pET 3a/Rosetta 2 (DE3) pLysS showed that this protein could be the required hypothetical phage protein from *Campylobacter* phage CP220 of predicted mass 26.3 kDa. The NCBI search output from the MASCOT database is shown in Figure 6.7. An additional search using the semi-trypsinised protein identified an additional peptide for the phage protein (Figure 6.8). The MS tryptic peptide spectrum is shown in Appendix 6.



1. gi|294338091 Mass: 26295 Score: 278 Matches: 6(3) Sequences: 6(3)

hypothetical phage protein [Campylobacter phage CP220]

Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
500.2480	998.4814	998.4821	-0.0007	0	31	60	1	U	K.YQANYVNK.T
607.3414	1212.6682	1212.6714	-0.0031	0	75	0.0019	1	U	K.IQDLQIELNK.T
664.8852	1327.7558	1327.7500	0.0059	0	14	2.5e+03	2	U	K.QQSLILAEIWK.Q
665.2623	1328.5100	1328.5377	-0.0276	0	15	2e+03	4	U	K.EMSSMYHLSDK.Q+ Oxidation (M)
717.9147	1433.8148	1433.8071	0.0078	0	76	0.0014	1	U	K.INPAFLYAVLWK.E
742.9033	1483.7920	1483.7922	-0.0002	0	70	0.0055	1	U	K.NIEGTAYILSYLK.S

Match to: gi|294338091 Score: 278
hypothetical phage protein [Campylobacter phage CP220]
Found in search of 1A_MP0911_16March2011.pkl
Nominal mass (M_r): 26295; Calculated pI value: 9.86
NCBI BLAST search of gi|294338091 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Campylobacter phage CP220
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 29%
Matched peptides shown in Bold Red

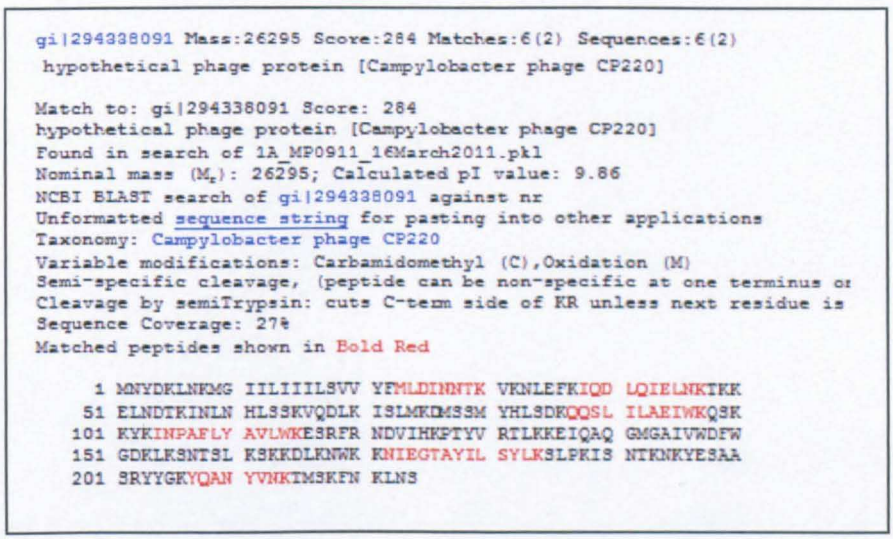
1 MNYDKLNKMG IILIIILSVV YFMLDINNTK VKNLEFKIQD LQIELNKTKK
51 ELNDTKINLN HLSSKVQDLK ISLMKDMSSM YHLSDKQQSL ILAEIWKQSK
101 KYKINPAFLY AVLWKESRFR NDVIHKPTYV RTLKKEIQAQ GMGAIVWDFW
151 GDKLKSNTSL KSKKDLKNWK KNIEGTAYIL SYLKSLPKIS NTKNKYESAA
201 SRYYGKYQAN YVNKIMSKFN KLNS

2. gi|15803846 Mass: 22073 Score: 67 Matches: 1(1) Sequences: 1(1)

50S ribosomal protein L4 [Escherichia coli O157:H7 EDL933]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
65	781.4109	1560.8072	1560.8035	0.0037	0	67	0.012	1	U	R.DATGIDPVELIAFSK.V

Figure 6.7 NCBI search results of the expressed CP220 CPT 120 protein band tryptic digest



gi|294338091 Mass:26295 Score:284 Matches:6(2) Sequences:6(2)
hypothetical phage protein [Campylobacter phage CP220]

Match to: gi|294338091 Score: 284
hypothetical phage protein [Campylobacter phage CP220]
Found in search of 1A_MP0911_16March2011.pkl
Nominal mass (M_r): 26295; Calculated pI value: 9.86
NCBI BLAST search of gi|294338091 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Campylobacter phage CP220
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus or
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is
Sequence Coverage: 27%
Matched peptides shown in Bold Red

1 MNYDKLNKMG IILIIILSVV YFMLDINNTK VKNLEFKIQD LQIELNKTKK
51 ELNDTKINLN HLSSKVQDLK ISLMKDMSSM YHLSDKQQSL ILAEIWKQSK
101 KYKINPAFLY AVLWKESRFR NDVIHKPTYV RTLKKEIQAQ GMGAIVWDFW
151 GDKLKSNTSL KSKKDLKNWK KNIEGTAYIL SYLKSLPKIS NTKNKYESAA
201 SRYYGKYQAN YVNKIMSKFN KLNS

Figure 6.8 The semi-tryptic digest NCBI search result of the expressed CP220 CPT 120 protein band.

* The matched peptides are shown in red

6.2.6 Effect of IPTG on Bacterial Growth

The effect of induction with 0.1 mM IPTG on the cell growth of CP220 CPT 120 cloned in pET 3a and expressed in Rosetta 2 (DE3) pLysS was studied using expression clones cultured for 12 h with the OD 600 readings being taken on an hourly basis (see method section 2.8.9). The induction was initiated after 3 h incubation at 37 °C, 100 rpm when the OD 600 was between 0.3-0.4. The cells continued to multiply for 4 h after which the cell concentration started to decrease until the end of the incubation period (Figure 6.9).

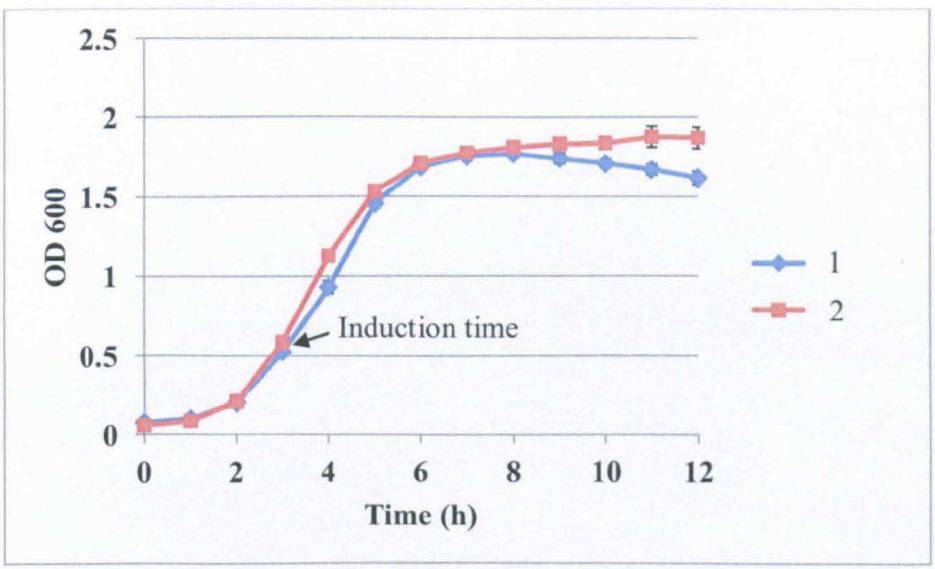


Figure 6.9 Effect of IPTG on *E. coli* Rossetta expressing CP220 CPT 120 growth

1: pET 3a/Rosetta 2 (DE3) pLysS Expressing CPT 120

2: pET 3a/Rosetta 2 (DE3) pLysS (empty vector control)

6.2.7 Isolation of Cell Fractions

Cells from cultures exhibiting a protein of the estimated size (see section 6.2.3) were fractionated into periplasmic space, cytoplasm and membrane proteins and inclusion bodies to assess the location of the protein (see method section 2.8.8). These fractions were examined by 10-20 % Novex Tricine gels as described in methods section 2.8.10 (Figure 6.10). The isolated cell fractions from *E. coli* expressing CP220 CPT 120 in pET 3a/Rosetta 2 (DE3) pLysS showed that the majority of the expressed protein was present in the cytoplasm (lane 4) even though there was a degree of contamination in the membrane protein and the inclusion bodies fractions (lanes 5 and 6).

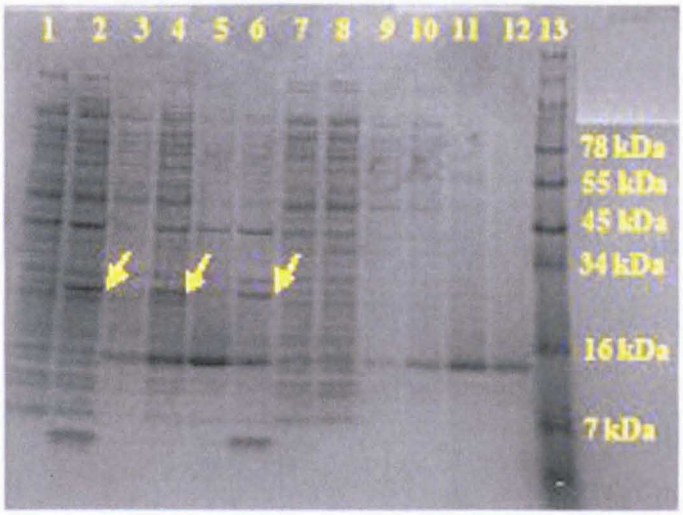


Figure 6.10 Cellular fractions of *E. coli* Rosetta 2 (DE3) pLysS expressing CP220 CPT 120 from pET 3a

To assess the location of the expressed protein within the cell, different fractions were isolated pre- and post-induction with 0.1 mM IPTG and electrophoresed together with a negative control on a 10-20 % tricine gel. Lanes representing the isolated fractions are as follows:

Lanes 1-6 expressed CP220 CPT 120

Lanes 1-2; total cell fraction, lanes 3-4; cytoplasm; lanes 5-6; membrane proteins and inclusion bodies.

Lanes 7-12 pET 3a/Rosetta2 (DE3) pLysS (negative control)

Lanes 7-8; total cell fraction, lanes 9-10; cytoplasm; lanes 11-12; membrane proteins and inclusion bodies, lane 13; pre-stained See blue plus 2protein marker.

* First lane of each fraction was pre induction and the second lane was after 4 h of induction with IPTG.

6.2.8 Testing Lytic Activity of CPT 120

The lytic activity of recombinant CP220 CPT 120 protein was tested against a number of strains including; *Campylobacter* PT14, *E. coli* 3110 and *Salmonella* Typhimurium DT 104 NCTC 13348 as described in methods section 2.11. The *C. jejuni* PT14 plates were incubated at 42 °C under microaerobic conditions whereas *E. coli* and *Salmonella* plates were incubated at 37 °C aerobically on NZCYM medium.

Dispensing droplets of a protein fraction containing protein CP220 CPT 120 on to the confluent plates of *C. jejuni* PT14 resulted in an area of lysis. A similar pattern of lysis was found for *E. coli* 3110 and *Salmonella* Typhimurium DT 104 NCTC13348. The central regions of the lysed areas exhibited eventual re-growth of the bacteria either due to binding and loss of protein function or possibly due to the development of resistance. The area of lysis produced was too diffuse to produce an image of sufficient quality to include but was clearly visible with the naked eye.

6.2.9 His Tag Addition to CPT 120

Oligonucleotides were designed to add a six histidine protein tag as an N-terminal translational fusion (His tag) to CP220 potential lysin encoding gene CPT 120 (Table 6.2). The histidine residues were added to enable one-step purification of the protein product using metal affinity chromatography.

Table 6.2 His Tag Oligonucleotides

Oligonucleotide Name	**Sequence**
His Tag: F	5'-T ATG CAC CAC CAC CAC CAC CA-3'
His Tag: R	5'-T ATG GTG GTG GTG GTG GTG CA-3'

DNA from the plasmid containing the CPT 120 gene in pET 3a was prepared and digested using *Nde*I before dephosphorylation using antarctic phosphatase as described in methods sections 2.10 and 2.8.6.2 respectively. The oligonucleotides (His Tag) were annealed and ligated into the vector (CPT 120 in pET 3a in *E. coli* TOP 10 cells) at various ratios of insert:vector (3:1, 10:1, 30:1) using T4 DNA ligase (Promega) as described in methods section 2.8.7.4. The ligation mixture was transformed into chemically competent *E. coli* TOP10 cells before plating on LB agar plates containing ampicillin (2.4.1) to select for the plasmid (described in methods sections 2.8.3 and 2.1.5).

Colonies obtained were tested by direct DNA amplification using primers specifically designed to have the His tag together with a reverse primer designed for CPT 120 (Table 6.3).

Table 6.3 Primer sequences to amplify the His tagged CP220 CPT 120

CP220 Gene	Position	Sequence
His Tagged CPT 120	107127... 107801	F: 5'-AA CAT ATG CAC CAC CAC CAC CAC CAC ATG AAT TAC GAT AAA CTG AAT AAA ATG GG-3' R: 5'-AAG GAT CCG TAT TAA GAG TTT AGT TTA TTA AAT TTT GAC-3'

Bacterial DNAs were liberated by boiling and used as a template for PCR using the following cycles:

1 cycle	96°C	3 min
35 cycles	96°C 65°C 72°C	1 min 1 min 1 min
1 cycle	72°C	7 min

PCR amplification products were examined using 0.8 % agarose gel electrophoresis and the gel image recorded on a ChemiDoc XRS Imager using the Quantity one program (Bio-Rad). However, none of the PCR products resulted in the required band (data not shown).

6.2.10 CP220 CPT 120 PCR Amplification with alternate His Tagged Primers and Cloning

As an alternative strategy, the reading frame of CPT 120 was PCR amplified with the original primers carrying a His tag (Table 6.3) at the *Nde*I site as described in section 4.2.5, and the DNA product cloned into the plasmid vector pCR 2.1-TOPO, transformed into *E. coli* TOP10 chemically competent cells and the plasmid selected on LB culture plates containing ampicillin (2.4.1) as described in the methods (see sections 2.8.2 and 2.8.3). Plasmid DNAs were prepared from ten colonies, and digested with *Eco*R1 (described in methods sections 2.8.4 and 2.8.5). Of these eight had insert sizes consistent with the target DNA amplicon (Figure 6.11), from which six were selected for DNA sequencing and the insert sequences analyzed using BioEdit. Four sequenced clones were identical to CPT 120 and His tagged successfully. The DNA sequence of the His tagged CPT 120 gene is shown in Appendix 7. DNAs encoding the His tagged CPT120 were cut from the pCR 2.1-TOPO plasmid using *Nde*I and *Bam*HI and subcloned in pET 3a and pET 11a as described in methods section 2.8.6. However, none of the diagnostic digests indicated that the gene had been cloned in either pET 3a or pET 11a.

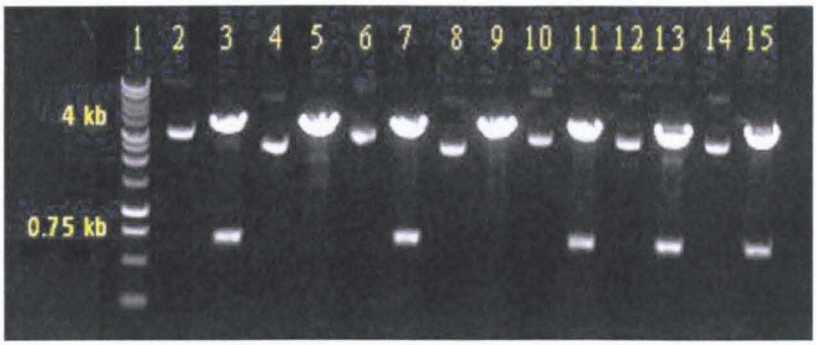


Figure 6.11 Restriction digests of His tagged CP220 CPT 120 plasmid clones in pCR 2.1-TOPO

In order to check the presence of the correct insert in pCR2.1-TOPO, plasmids were prepared, digested with *Eco*RI and analyzed by electrophoresis on a 0.8 % agarose gel. Lanes representing these plasmids are as follows:

Lane 1; 1 kb marker, lanes 2-3; colony 1, lanes 4-5; colony 2, lanes 6-7; colony 3, lanes 8-9; colony 4, lanes 10-11; colony 5, lanes 12-13; colony 6, lanes 14-15; colony 7

* First lane of each sample was undigested plasmid; the second lane was digested with *Eco*RI

6.3 Discussion

The bacteriophage CP220 open reading frames CPT 0075 and CPT 120 were PCR amplified and cloned successfully into pCR2.1-TOPO vectors and subcloned into pET 3a and pET 11a (CPT 0075 only) using *E. coli* cells that will not permit expression of the genes from the T7 promoters of the pET vectors.

Transformation of the cloned genes present in the pET plasmids into an *E. coli* expression host carrying T7 RNA polymerase was challenging. BL21 (DE3) strains were the first choice of hosts because they carry an IPTG-inducible T7 RNA polymerase in a genetic background deficient in both *lon* and *omp*T proteases. This occurs because the *E. coli* host contains the λDE3 lysogen that carries the T7 RNA polymerase gene as a stable chromosomal copy, which is controlled by *lac*UV5. Optionally the pLysS plasmid can be used that carries the gene encoding T7 lysozyme, which can lower any background expression of the target genes as it is controlled by the T7 promoter without interfering with the expression level following IPTG induction. The host BL21 (DE3) pLysS did not express either CPT 0075 or CPT 120 and was substituted with BL21 (DE3) pLysE, which provides tighter control of T7 RNA polymerase. This is an important consideration when the required protein is potentially toxic to the host. However, none of the genes could be expressed in this host strain.

The expression host was changed to Rosetta 2 (DE3) pLysS, which is a derivative of *E. coli* BL21 known to enhance the expression of eukaryotic proteins since it contains tRNAs for codons rarely used in *E. coli*. Such cells provide tRNAs for seven rare codons: AGA, AGG, AUA, CUA, GGA, CCC and CGG. Rosetta 2 cells were the host choice and the protein product of CPT 120 was successfully produced in this background based on the identification of tryptic peptides using mass spectrometry.

Protein expression could have been problematic for a number of reasons. One of the problems could be the incompatible codon usage of CP220 attuned for expression in *Campylobacter* species with low GC contents. Each amino acid can be encoded by more than one codon and different organisms prefer certain codons that often reflect the GC content of the genome. Such codon usage is affected by the availability of tRNAs in the organism and some phages carry tRNAs in their genomes, potentially to compensate for such compositional differences (Bailley-Bechet *et al.*, 2007). However, the most likely cause of these difficulties is that the product has been selected to encode a putative lysin that will disrupt the host following its induction. The codon usage analysis for CPT 120 and 0075 was performed and showed that rare codons present in Rosetta 2 (DE3) pLysS were used in a percentage of 3.5 and 8.2 % for CPT 120 and 0075, respectively. These rare codons in this host did not have any effect on CPT 0075 expression while their use for CPT 120 resulted in successful expression.

Alternative expression systems that do not rely upon a potentially sensitive bacterial host may be appropriate for lysin production, expression systems such as the methylotrophic yeast *Pichia pastoris* are possible candidates (Daly and Hearn, 2005).

Maximum expression of gene CPT 120 was achieved after 4 h of induction with 0.1 mM IPTG. The lytic activity of the CPT 120 protein expressed in this manner was tested on the Gram-negative bacteria: *Campylobacter jejuni*, *E. coli* 3110 and *Salmonella* Typhimurium DT 104. This lysin was originally amplified from phage CP220 that is capable of lysing a wide range of C. *jejuni* and *C. coli* strains (El-Shibiny *et al.*, 2009) with a view to generating a similarly wide ranging lysin activity. The CPT 120 protein showed some level of lysis with bacterial species other than *Campylobacter*. However, the area of lysis was subject to re-growth of bacteria, which could be due to the loss of protein activity due to binding to cell wall substrates from lysed cells or possibly due to the development of resistance.

The spot test of lytic activity was a crude preliminary test of lytic activity and it indicated that potential lytic activity needs further investigation. This could only be done properly once the His tag problems are rectified, allowing larger quantities of pure lysin to be expressed. Further experiments would have to be carried out using many more strains and species, and using different approaches such as comparing viable counts before and after treatment.

When the His tag was added to the primers used for gene CPT 120 PCR amplification, the gene was amplified and cloned in pCR2.1-TOPO vector successfully using *E. coli* TOP10 chemically competent cells, but technical problems resulted in failure to subclone this gene into the pET system, although it had previously been cloned in it without the His tag. This last hurdle needs further attention because the His tag will provide a means to purify the protein away from cellular debris that could affect the function of the lysin.

The presence of the outer membrane in campylobacters is a barrier that prevents lysins from accessing their cellular targets and lysing the bacteria directly. To prove the activity of the prepared CPT120 lysin, the outer membrane should be permeablized prior testing this lysin on campylobacters and other Gram negative bacteria.

In a study performed by Walmagh *et al.* (2012), five endolysins from phages targeting the peptidoglycan cell wall of Gram negative bacteria were examined. These bacteria included: *Burkholderia cepacia*, *E. coli*, *Salmonella enterica* and *Klebsiella pneumonia*. The lysins were tested individually and in combination against *Pseudomonas aeruginosa* PAO1 (wild type), *Pseudomonas aeruginosa* Br667 (multidrug resistant burn wound isolate), *E. coli* XL1-Blue MRF and *Salmonella enteric* a Serovar Typhimurium LT2 but did not result in any lysis. On the other hand, when the work was repeated in the presence of EDTA which permeablized the outer membrane, the lysins proved to be effective, lysing all the bacteria tested.

CHAPTER SEVEN

DISCUSSION

The work in this thesis describes the isolation and comprehensive characterization of bacteriophage candidates for future therapy applications to reduce *Campylobacter* contamination of poultry.

Efforts to extend the number of available phages for phage therapy were made and three new phages were isolated, two of which (4a and 18b) were characterized with ten previously uncharacterized phages from our laboratory stocks. The two new phages were found to belong to class III of the *Campylobacter* phages and could be demonstrated to infect and lyse campylobacters *in vitro*. Their activity against campylobacters colonizing chickens *in vivo* needs to be investigated.

All the phages characterized had different lytic profiles with no single phage able to lyse all the *Campylobacter* strains tested. To overcome this problem, a high number of phages should be tested to find ones that are effective against a wide range of pathogenic strains. A solution that can be applied in parallel is the use of a cocktail of phages to increase the effective host range and to target different surface recognition molecules and receptors, and thereby reduce the chance of the survival of *Campylobacter* phage resistant strains (Carlton, 1999). From this work, candidate phages can be mixed and tested as a phage cocktail against campylobacters from different sources. For example, the cocktail could include the newly isolated phage 18b together with CP20 and CP30, as these phage showed a broad spectrum of activity against range of campylobacters compared to the other phages examined. Carvalho *et al.*, (2010a) reported the use of three lytic phages against *C. jejuni* and *C. coli* in chickens, where they were tested using two routes of administration (oral and

feed) to successfully reduce colonization by 2 $\log_{10}$ CFU g^{-1}. Similarly, a number of studies have been performed for the control of other bacteria using phage cocktails. The control of *E. coli* infection and contamination using phage has been reported in different species including calves (Smith and Hugens, 1983), beef meat (O'Flynn *et al.*, 2004) and mice (Tanji *et al.*, 2005). Moreover, *Salmonella* reduction in pigs and pork (Zhang *et al.*, 2010 and Hooton *et al.*, 2011), and biofilm formation by *Pseudomonas* on catheters (Fu *et al.*, 2010) have all been successfully treated using phage cocktails.

Phages can be found in niches where their hosts exist and proliferate. *Campylobacter* phages can be isolated from a number of sources including: chickens (Atterbury *et al.*, 2003a and Connerton *et al.*, 2004), pig manure (Khakhria and Lior, 1992), sewage (Salama *et al.*, 1989; Beaudoin *et al.*, 2007) and slaughter house effluent (Salama *et al.*, 1989). One example of phages isolated from chicken is phage W5, which was isolated from a chicken carcass and belongs to class III of the *Campylobacter* phages (Atterbury *et al.*, 2003a). In this study, W5 was characterized through its lytic profile, and its genome size was estimated using PFGE. The genomic DNA of W5 showed two bands when analysed by PFGE (a major band corresponding with a genome size of approximately 130 kb and a minor band estimated at 195 kb), implying the presence of two genomes, despite plaque purification. Attempts were made to separate these two genomes to investigate any potential genetic relationship. These attempts resulted only in the recovery of the phage with the smaller genome, possibly due to the greater representation of this class of phage in the sample. The observation that *Campylobacter* phages are recovered as co-

isolates has also been recently reported from chicken faeces in Australia. In this report a genome estimated at 195 kb was observed together with one of about 140 kb, which remained associated after plaque purification (Owens *et. al.*, 2012).

Attention was further focussed on a second pair of co-isolates, which had been successfully separated, with the sequence of larger genome, CP220, having been determined previously (Timms *et al.*, 2010). The co-isolate of CP220, CPX, is a class III phage with a genome somewhat smaller than CP220 which belongs to class II. These phages have different lytic profiles, thus they could potentially be of a greater therapeutic value when mixed together. Moreover, they have limited sequence similarity at the nucleotide level, indicating that there is no extended sequence relationship between them, despite their co-isolation. The distinction between their activity against *Campylobacter* strains and the fact that they are genetically diverse make them complementary candidates for inclusion in phage cocktails. The use of diverse phages with different receptor requirements and functional activities against the target bacteria will limit the potential for the development of phage resistance, since more than one mutation in the bacterial population may be required to overcome infection by different bacteriophage. These mutations must also retain the fitness of the bacteria to compete with resident intestinal microflora and survive in the wider environment.

However, in the context of co-isolating phages, further work will be required to investigate the relationships between the phage, and whether they will co-infect permissible host bacteria or gain stability by association with each other. The maintenance of genetically distinct phages upon serial plaque passage clearly challenges the generally held belief that individual plaques represent individual phage clones. The fact that other examples of co-isolation exist, such as W5 described in Chapter 3, indicates that this is not an isolated phenomenon and deserves further investigation.

The molecular comparison of *Campylobacter* phage genomes became easier with the availability of phage CP220 genome (Timms *et al.*, 2010). PCR amplification of different phage DNAs based on primers designed on selected genes from phage CP220 enabled their comparison, and showed that among all the amplified genes, CPT 120, which this thesis has identified as a potential lysin, is present in all the phages examined, either as identical sequences or with few sequence differences that are either silent or lead to conservative changes and are not likely to change the function of the product protein. The lysin appears conserved throughout the different types of *Campylobacter* phage, which makes it an antimicrobial agent with potentially wide application (Chapter 4). This thesis has further demonstrated the lytic activity of CPT 120 as a crude lysin against other Gram-negative bacteria including the foodborne pathogens *E. coli* and *Salmonella* Typhimurium (Chapter 6).

This lysin gene was cloned and successfully expressed in the pET system of *E. coli*, yet DNA constructions aiming to add a histidine tag to enable rapid purification of the lysin were not successful (Chapter 6). To enable further

characterisation of the lysin it will be necessary to purify the protein, and if the lysin was to be used for any biotechnological application this would be essential. Affinity purification kits for histidine-tagged proteins are available from different commercial sources including: Qiagen, Sigma, Thermo Scientific, GE Healthcare, Bio-Rad and others. These kits are convenient, efficient, inexpensive and have been demonstrated to produce high yields of purified tagged proteins. If the pET system was to prove intractable for expression of phage proteins then a different system should be sought. For example, *Pichia pastoris* is an excellent expression system that may not have some of the drawbacks evident for a Gram-negative bacterium producing an active lysin. As a methylotrophic yeast, *Pichia pastoris* could be capable of the expression of phage proteins as they may not interfere with cell metabolism and growth as they are programmed to do for bacteria. Yeast systems benefit from prokaryote-like ease of handling and have robust well developed fermentation systems (Daly and Hearn, 2005).

Determination of the genome sequence of phage CPX was undertaken for two reasons: firstly developments in sequence technology for the first time allowed the sequence determination of a group III *Campylobacter* phage, as at the time of starting this work, no complete genome sequences from this group were available. Secondly this phage was co-isolated with CP220 and any relationship at the sequence level between these two phages could only be investigated by having the sequences to compare. The sequence determination of CPX was finished around the same time that two further group III *Campylobacter* phage sequences became available (Hammerl *et al.*, 2011;

Kropinski *et al.*, 2011). Sequencing phages is increasing worldwide. This will allow the comparison of different phages to understand their relationships and to investigate the presence of similar genes of similar potential functions in different phages. The sequence of CPX revealed many interesting features. However, a large proportion of the putative CDSs like CP220 and CPt10 showed no significant matches to available sequences in data bases (Timms *et al.*, 2010).

Like other *Campylobacter* phages, homing endonuclease encoding genes (*Hef*) were identified in the CPX sequence (Timms *et al.*, 2010). Of the group III phage, CPX has seven Hefs compared with NCTC12673 that has twelve in total (Kropinski *et al.*, 2011), whereas CP81 has eight homing endonucleases (Hammerl *et al.*, 2011). Although CPX phage has nineteen genes that are similar to T4 phage, it is not classified together with the other class III phages as T4-related because it is smaller than the smallest characterized T4 genome. The genes that share protein sequence identity with T4 phage structural genes are scattered throughout the phage genome and are listed in chapter 5. The exception to this is a short gene cluster of three ORF's that lie on the negative strand and preserve the corresponding T4 order of gp20 (portal vertex protein), gp21 (prohead protease) and gp23 (major capsid protein). The T4-like modules would imply functional conservation of a virulent lifestyle but possibly without the need for temporal transcription units.

In summary, this thesis describes a closer examination of the molecular biology of *Campylobacter* bacteriophage than had previously been possible, significantly extending our knowledge in this area. Of particular importance is the determination of DNA sequence of CPX phage and the identification of an active potential lysin gene product from the CP220 DNA sequence.

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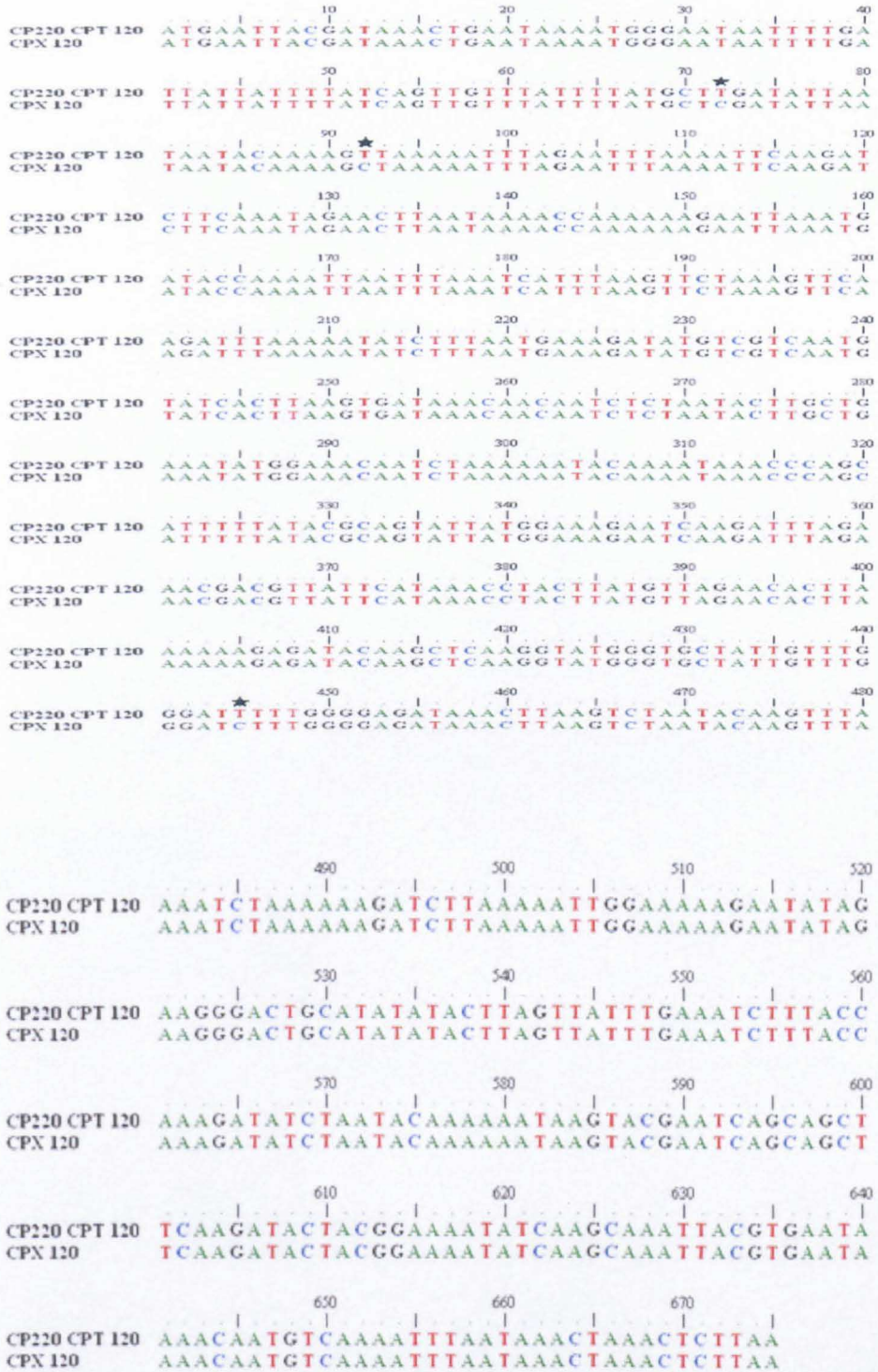
APPENDICES

Appendix 1

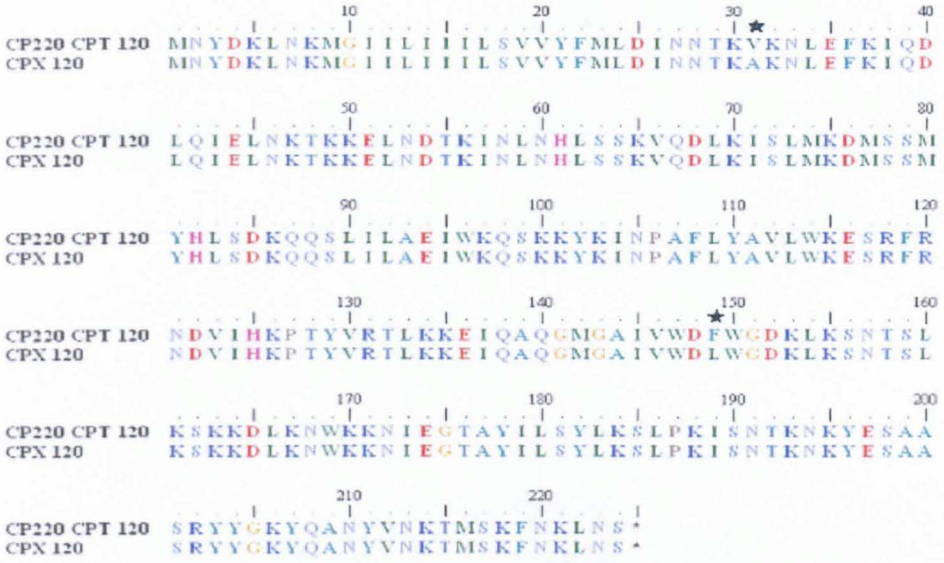
Comparison of the nucleotide sequences of genes CP220 CPT 0075 and CP20 0075 where the differences between the two sequences are indicated with star symbols

Comparison of the predicted amino acid sequences of genes CP220 CPT 0075 and CP20 0075 where the differences between the two sequences are indicated with star symbols

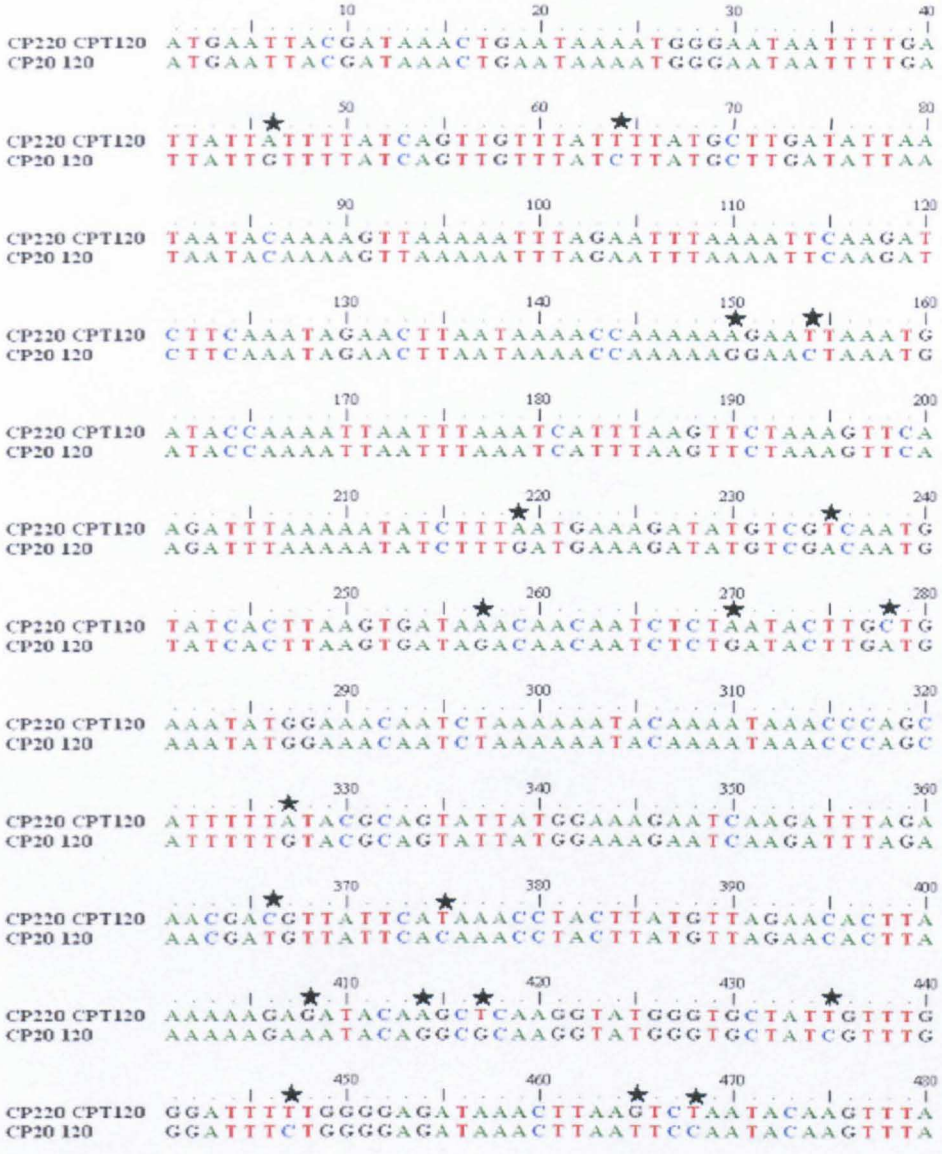
Comparison of the nucleotide sequences of genes CP220 CPT 120 and CPX 120 where the differences are indicated by star symbols

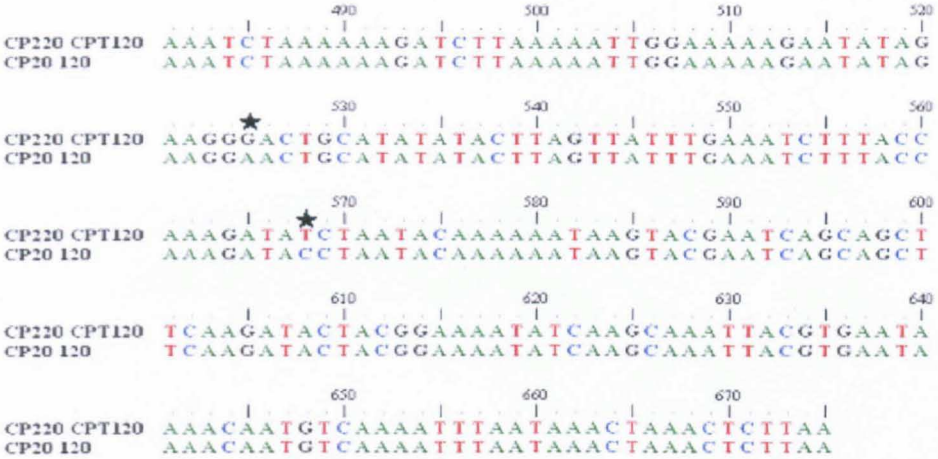


Comparison of the predicted amino acid sequences of genes CP220 CPT 120 and CPX 120 where the differences are indicated by star symbols

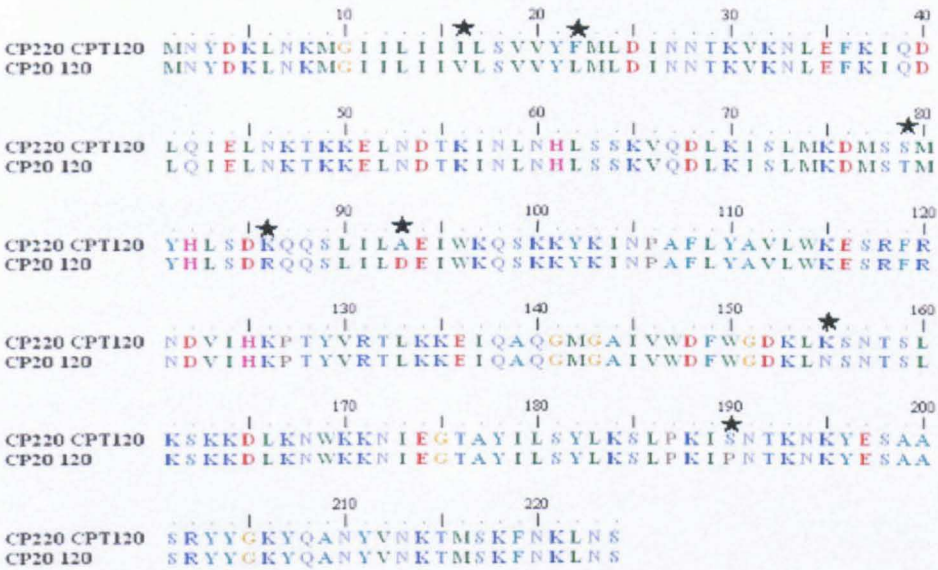


Comparison of the nucleotide sequences of genes CP220 CPT 120 and CP20 120 where the differences are indicated by star symbols

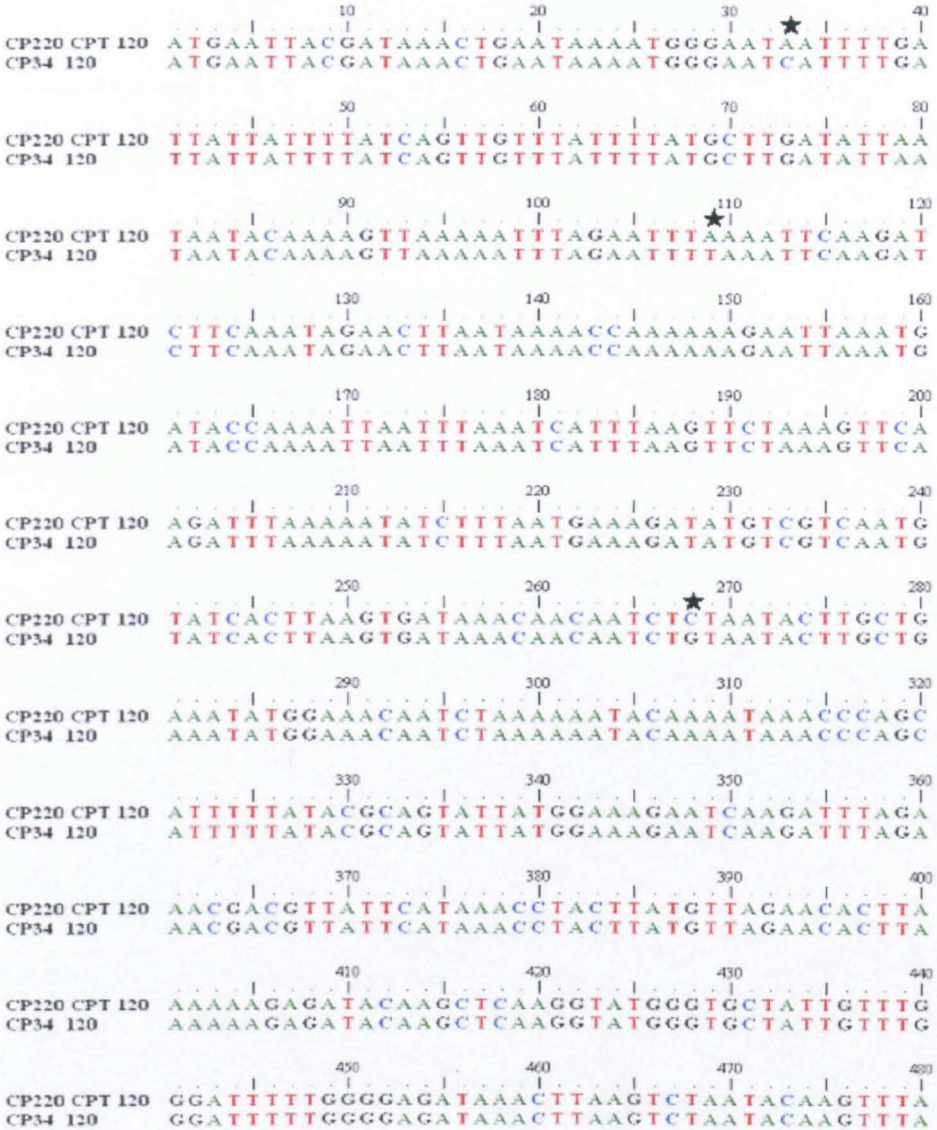


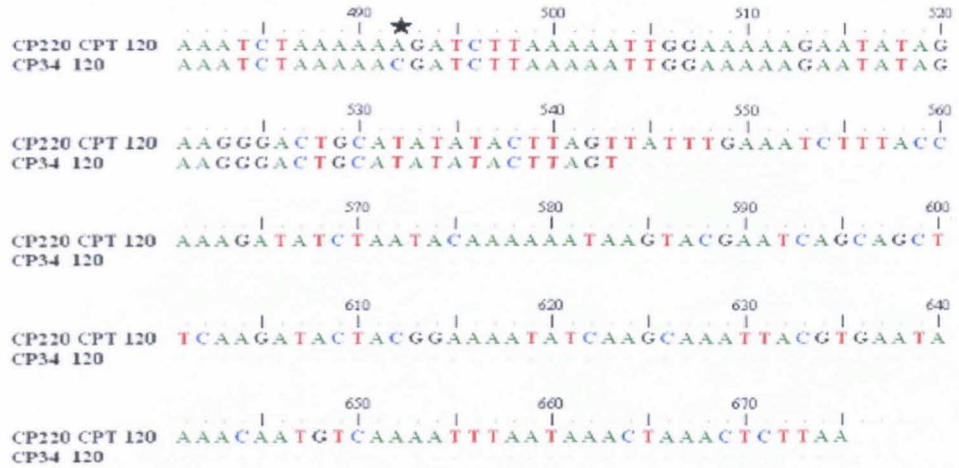


Comparison of the predicted amino acid sequences of genes CP220 CPT 120 and CP20 120 where the differences between the two sequences are indicated with star symbols

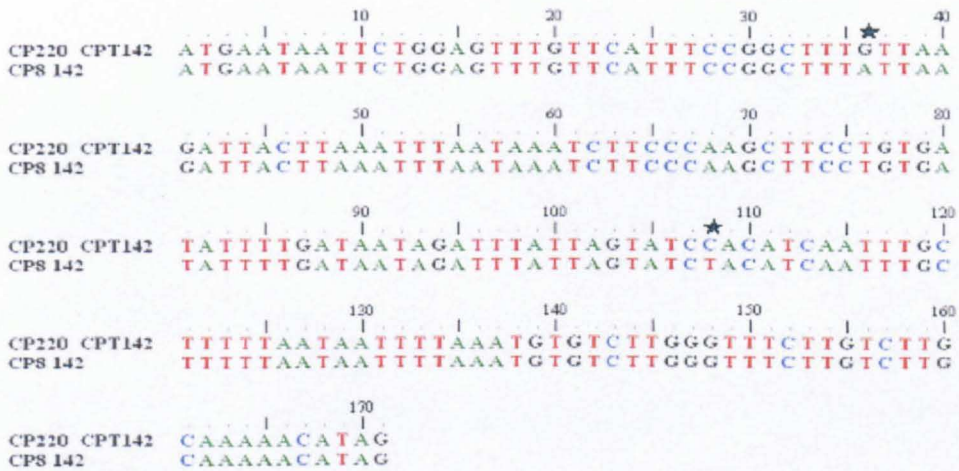


Comparison of the nucleotide sequences of genes CP220 CPT 120 and CP34 120 where the differences are indicated by star symbols





Comparison of the nucleotide sequences of genes CP220 CPT 142 and CP8 142 where the differences are indicated by star symbols



Nucleotide sequence of gene CP220 CPT 0045 with the amplified region shown in blue

ATGGCTGATATTATTCAAAATAAAATGAATCAAGTACTAGGTGATTTAGC
TCGTCCCACTAAGTTTAAATGTCAAATATTTCCACCTAAAGAAATTAAGTG
TGAATTGAGTATTTTAAATGAAGGTGATTCTGCAACATCCAGCACTTCTGA
AATAAGCCAATATTTAGACTATTTTTGTCACGCTACAAGTTTTCCGGGATT
GACTGTAGAAACAATAGATTTTAAATATCGTGGTAGGACTCTACCAGTTA
AATCAGTACAAACTTATCAACAAAAATGGTCAGCAACTTTTTATAATGAT
GAAAAACATGCAGTTAGAAAGTTATTTTTGGATTGGATGACTTATGATCA
AGCACATCAATTTGAGGATAAAACTAAAGGTAATTTTGAAGGTATATTAC
CCAGCATTTCTATATATCAATTAGATTTTGAAATGTCTAAAGATTGTGTTG
TATATACTATGATGAATGTATTTCCAACAAATGTGGGAGAAATTTCAGTTC
AATATGACGGGTTAAATCAAATTGAAACTTTTACAGTTGAGTTTGCATATA
CCCATTTTGAAATTAATACAATTTCTAGGGAAGGGTTAACGAGTTCTGAA
GTCACTAGTTTGATTAAGAATACTATACAGAATACTATTAATAATGTTACC
AATACTTTAAAGGATGCTGTATTTGGTGCTTTAGACGACCTAGTTTCACCT
GTATTGGATTCGGTTTCAGATTCATTTGAAAATTTTATAAGTACAAAATAA

Predicted amino acid sequence of gene CP220 CPT 0045

MADIIQNKMNQVLGDLARPTKFKCQIFPPKEIKCELSILNEGDSATSSTSEISQY
LDYFCHATSFPGLTVETIDFKYRGRTLPVKSVQTYQQKWSATFYNDEKHAVR
KLFLDWMTYDQAHQFEDKTKGNFEGILPSISIYQLDFEMSKDCVVYTMMNV
FPTNVGEISVQYDGLNQIETFTVEFAYTHFEINTISREGLTSSEVTSLIKNTIQNT
INNVTNTLKDAVFGALDDLVSPVLDSVSDSFENFISTK

Nucleotide sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0045 sequence with the differences to CP220 CPT 0045 sequence shown in red

CAAGTTTTCCGGGATTGACTGTAGAAACAATAGATTTTAAATATCGTGGTA
GGACTCTACCAGTTAAATCCGTTCAAACTTATCAACAAAAATGGTCAGCA
ACTTTTTATAATGATGAAAAACATGCAGTTAGAAAGTTATTTTTGGATTGG
ATGACTTATGATCAAGCACATCAATTTGAGGA

Nucleotide sequence of gene CP220 CPT 0058 with the amplified region shown in blue

ATGTCTAACAAAATTGAAGAAATTAAAACCGCACTAAAATCTGGTGCAAA
AGCTACAAAATACCGTGTTAAACTTTCATTTCCAACAGAAGTGCAACATA
AAATGGAATTACAAAGCTTGAACTGCTTAGCTAAAGCTACTAGTTTTCCA
GGTGTAACTATTGGACAAATTGAAGTATTTAACCAAGGAAGAAAGCTTCC
TATACCTGGTGATACTTCGTATGATACACAATGGACCGTAACATTTTATAT
GGATAATGCACACCAAACTCGTAAAGACTTCTTAAGTTGGATGAAAGCTT
GTGACAACTTCCAAGCAAATACCCATTCTGGTAACCCAGGGGGCTTATTT
ACAGAAGTTTCGGTTTGTCAATTAGATTCATTAGAAAATGAAGTTGCTGA
ATATACTTTAAGAAACTGCTGGCCGAGTGGTGTTGGTGAAATTAGTGTTG
GTGCTGATCAATTAGATACATTACAAGAATGTGATATCACATTTAGCTTCT
CAGATTGGATTATTTCTAATGGATCTGAATTTAATATGCCACAAGATGGTA
AATCGGCTGCTACTAACGTAGTTTCTGTAGACCAATAA

Predicted amino acid sequence of gene CP220 CPT 0058

MSNKIEEIKTALKSGAKATKYRVKLSFPTEVQHKMELQSLNCLAKATSFPGV
TIGQIEVFNQGRKLPIPGDTSYDTQWTVTFYMDNAHQTRKDFLSWMKACDN
FQANTHSGNPGGLFTEVSVCQLDSLENEVAEYTLRNCWPSGVGEISVGADQL
DTLQECDITFSFSDWIISNGSEFNMPQDGKSAATNVVSVDQ

Nucleotide sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0058 sequence with the differences to CP220 CPT 0058 sequence shown in red

ACCCAGGGGGCTTATTTACAGAAGTTTCGGTTTGTCAATTAGATTCATTAG
AAAATGAAGTTGCTGAATATACTTTAAGAAACTGCTGGCCGAGTGGTGTT
GGTGAAATTAGTGTTGGTGCTGATCAATTAGATACATTACAAGAATGTGA
TATCACATTTAGCTTCTCAGATTGGATTATTTCTAATGGGTCTGAATTTAAT
ATGCCACAAGATGGTAAATCGGC

Nucleotide sequence of gene CP220 CPT 0175 with the amplified region shown in blue

ATGGGTACTTTTTCATTTTCATTATCGGATATAAAGAAACAATTAGGTCCT
GGTTTAGGAGTTAGATCAAATGCTTACTTACTAGAAGTTGCTGTAGTAGGT
GCTGTTTCTAAAAAATTAGCAGTTCTTTGTCAAAGCACAGCGTTACCTGAA
AGAAATATTGGAACCACTGATATATTCTACAAAGGTAGAAAATATAAGAT
GCGAGGTGAAACAGACTTAAGTGGTACTTATACTATTAATATAACTGATG
ATTCTGAAATGAAACTTAGAAGAATGTTCGATAGCTGGATGAGAGAAGTG
GATAATACTACACCTAAAGGGACTAATGCTTTAGCAGGCTTATTTGGTGGT
GCTATGGGTGATTTAATGGAGGTAGCTAACGGAACTTTGAAAGCGGTTAA
TGAAATTAAATCTGCTTGGGAGTTTGATGGTGGTGTTTCTTGGCTTAAAAA
TATGATTATGGGCAAGCCACTGCCAGCAAATTATCAAACAACCGTAAATA
TTTGGCAATTAACTAAAGTCAAAGAAAAACTATATGGGTATGCTTTGACT
AATGCTTTTCCTATTGAAGTAGGTGCAGTAGAAGTTTCTGACGAAAATGA
AAATCAGTTATCTATGTTTAGTGTAACTTTTGCATATTCAGATTTTGAACCT
ATTGAAGATAAAGGTATAATCGGTCAAATAGTTGATACTGTAATAGGCCA
AGAAGGTCAAGAAATTGCACAAGGTGTTGAAAATCTATTGGATTAA

Predicted amino acid sequence of gene CP220 CPT 0175

MGTFSFSLSDIKKQLGPGLGVRSNAYLLEVAVVGAVSKKLAVLCQSTALPER
NIGTTDIFYKGRKYKMRGETDLSGTYTINITDDSEMKLRRMFDSWMREVDNT
TPKGTNALAGLFGGAMGDLMEVANGTLKAVNEIKSAWEFDGGVSWLKNMI
MGKPLPANYQTTVNIWQLTKVKEKLYGYALTNAFPIEVGAVEVSDENENQL
SMFSVTFAYSDFEPIEDKGIIGQIVDTVIGQEGQEIAQGVENLLD

Nucleotide sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0175 sequence with the differences to CP220 CPT 0175 sequence shown in re

AAGATGCGAGGTGAAACAGACTTAAGTGGTACTTATACTATTAATATAAC
TGACGATTCTGAAATGAAACTTAGAAGAATGTTCGATAGTTGGATGAGAG
AAGTAGATAATACCACACCTAAAGGGACTAATGCTTTAGCAGGCTTATTT
GGTGGTGCTATGGGTGATTTAATGGAGGTAGCTAACGGAACTTTGAAAGC
GG

Nucleotide sequence of gene CP220 CPT 0034c with the amplified region shown in blue

GTGCCAGTTCCAAGTGTTAGATTTATTGATAGTGATACCAGTTCAACATCA
AATTATTCACAACCAAGAAGAGTATTCTATGCTGGATATTTCGATAAAGG
TTCACCAGACACTTTAACACCCGTTTATTCTATATTAGATTTTAAAACAAA
ATTTGGTAAACCAAACAAAAATAATATAAATGACTGGTTTCAAATTTATA
ATTATTTTTTATATGATAATAATGAAATAGTTATTTCGAGATCTATCGGTG
AAAATTCAGTTAACGCAAGTATTAGCTATCCATTTAATGACTTTGATGTTA
GAATAGATAACTTAGATGATTTTAGAAATAAACCCATAATTTCTGAAAAT
AATTTCTTAAGAATCATTGCTAGAAACCCAGGAGAGTGGGGAAATGATTT
AACAGTTTGTATTTTTACACAATATGAAGTTCTTAATAATATGCTAATACA
CAGCAATTATTTAGCAAAAGATATTCAAAATTCAATGAGTTCAAATCAAT
ATTGTATTTGTGTGTTCTTAAAAGATACATTAATGGAAAAATATATCTTAA
AAGAATCTGAGGATATGGTAGATACTATTAATGAAAATTCTAATTATATA
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TAAAACAGTTTTTTATGGTTCTAATAGTCTTAAATTAAGTAACGGGTATGC
AAGTTTGCCAAGTACAACCCAAATAGATGAAACATATAAAAGTGTTGGTG
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TTATGTATTTGAAAATAATAATGAATATACAAGAAATGATTTAGCTTCAA
AAATAAAAGAAATTTGCAGAAGTTATGTTGCAGATAATAATATAGAAGAT
TTTAAAATAGTTTGTGATATTTCTAATAATCCTACTCAGGATAATAACATT
TATGTAGATGTTTATTATAAACCTAAATATTTAATTGAAGAAGTTGTATTT
AGAATTCAAGCAGCTAGTGAATTGCCTAGTTGA

Predicted amino acid sequence of gene CP220 CPT 0034c

VPVPSVRFIDSDTSSTSNYSQPRRVFYAGYFDKGSPDTLTPVYSILDFKTKFGK
PNKNNINDWFQIYNYFLYDNNEIVISRSIGENSVNASISYPFNDFDVRIDNLDD
FRNKPIISENNFLRIIARNPGEWGNDLTVCIFTQYEVLNNMLIHSNYLAKDIQN
SMSSNQYCICVFLKDTLMEKYILKESEDMVDTINENSNYIFIIFDPKKYKLYDG
NINYVDGLNRLADGNEPNSDKTVFYGSNSLKLSNGYASLPSTTQIDETYKSVG
ESNDYVFDFIIANTQSPNSAINLADTRGDCCAFIGIPRGIKPEEYIKQLQISNNA
VVYYGSKLQLNPFNNQNIYVNCIGDIVGLRTRLINSQELSVSHCKTIYSFLNTI
DLDIYLTESQIKDLYDLNNINIVKKGYSGIYALSENTLKGSKLTNRIIYFNLVREC
ENVALYYVFENNNEYTRNDLASKIKEICRSYVADNNIEDFKIVCDISNNPTQD
NNIYVDVYYKPKYLIEEVVFRIQAASELPS

Nucleotide sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0034c sequence with the differences to CP220 CPT 0034c sequence shown in re

GGGTATGCAAGTTTGCCAAGTGCAGCTCAAATAGATGAAACATATAAAAA
TGTCGGTGAATCTAACGATTATATATTTGATTTTATAATTGCTAATACACA
AAGTCCAAATTCTGCTATCAATTTAGCAGATACTCGTGGTGATTGTTGTGC
TT

Predicted amino acid sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0034c sequence with the differences to CP220 CPT 0034c sequence shown in red

VPVPSVRFIDSDTSSTSNYSQPRRVFYAGYFDKGSPDTLTPVYSILDFKTKFGK
PNKNNINDWFQIYNYFLYDNNEIVISRSIGENSVNASISYPFNDFDVRIDNLDD
FRNKPIISENNFLRIIARNPGEWGNDLTVCIFTQYEVLNNMLIHSNYLAKDIQN
SMSSNQYCICVFLKDTLMEKYILKESEDMVDTINENSNYIFIIFDPKKYKLYDG
NINYVDGLNRLADGNEPNSDKTVFYGSNSLKLSNGYASLPSAAQIDETYKNV
GESNDYIFDFIIANTQSPNSAINLADTRGDCCAFIGIPRGIKPEEYIKQLQISNNA
VVYYGSKLQLNPFNNQNIYVNCIGDIVGLRTRLINSQELSVSHCKTIYSFLNTI
DLDIYLTESQIKDLYDLNNINIVKKGYSGIYALSENTLKGSKLTNRIIYFNLVREC
ENVALYYVFENNNEYTRNDLASKIKEICRSYVADNNIEDFKIVCDISNNPTQD
NNIYVDVYYKPKYLIEEVVFRIQAASELPS

Nucleotide sequence of gene CP220 CPT 0052 with the amplified region shown in blue

ATGTGGTTCGAGAGACTCTTTGTAATATCAAATCATATTAAAAGGATTAA
AATGGCAAATTTACTTAGCCCTGGTATCCAGGTTTCTGAAGTAGATCAATC
TCAAATCACACCAGTTGAAGGTGACTCTGCTGCTGTTTTTGGTGGTGATTT
TGAGAAAGGACCTGTTGGTGTTCATACTTTAATCTCAAGTGTTCAAGAACT
TAGAGACAATTATGGTATGCCTAATACAAAGAATTACAACGATTACTATC
AAGTCCAAAATTTCTTAGCTTATAGCGGTGCAATCTATGTTTCGCGGGCAG
CTGATTTAAATGGGACGCCTACAAAATTAGACGGCTTACAATTTGAAGAA
AATGCATATAAAACAAATGTTAATGCTACTAAAGTTGAAGGTGTTAAAGT
TATCGAAGCTGACTCTGTAGACGTTAAATTCGAAAAAACTGATAAATTTC
AAGTTGGTCAAGTTCTTAAATTCAACGATTCTAACAAAGAATATAAAATT
AAATATGTTAGAAACGAAGTTAAACAAATACCAAACCCAGATTATCAACC
ATTAACACAATTAGTAGTAGATCCAAGCCAAGCAAGTGCTTATGTTGATG
AAGTTGTTAGCTACGTAGTAACAACCAATGCAGAATCTTATACTGTAGAA
ACAGACAGGCCTGATGTAGTTCTTGTTAATAAATCTAATAAATCTTTAACT
GCATTAAAAGTAGGAACTGCTATTGTAACTTTTAGAGCTACAAAAGAAGG
TTCAAGGCCAAATACTTTTGAATTTGTTTTAAATGTTCAAGAAAAAGAACA
AACTAAGCTAGTAGTAACACCAGAAACTGTAAATATTTTAGAAGGACAAA
CTGCAATGCTTAATATAGATACAGATGCTGAAACTTATAGTATAGTATCTA
AAAACTTAGCAATAGCAACCATAGCCGAAGATAAAAAAACTATCAATGG
TTTGAGAATAGGTTCTTGTTTAGCCGAAGTATCAGCACAAGCTAATAATA
AAACAGAAACTACTAAAATCATAAATGTCAATGTTATCACAGGTGTTGAA
ACAGATTTAACAGTAAGCCCAGAAGGACCTGTAACTTTACACAAAAACGA
AGAACAAATATTTGAAATCACTTCCAGTGGGGCTTCTATATTAGAAACTG
CTAGTGAAGCTGATAAAGAATTTGTAACCGTAGACAAAACTGCTAAAAAA
GTTACTGCAATAAAAGAAGGTAATGCTGTAGTCCGAGTCCGCGCAAAAGC
TTTAGGTGCTGATGAAGTTATAAAAAGAATTCAAATAATTATTTTACCAGA
AAAAATAAGTGCCACTGTTGAGCCTACTGAAATAAGTATTAATACAGACG
CTGGCGACCAAACCTTAACGGTAACAACTGAAGCTACAAATATATCTGCA
CGCGTTATAGATACTTCAGTCGCAAATGTGAGCACCCAAGAAAAAATAAT
TACTGTAACACCATTAGCTGCTGGTAATACAGAGATTGAAATAACTGTTTC
ATCAGAAGGTTATTCAAATAATGTTATCACTGTTCCTTTAACAGTTACTGC
TGTAAATGCAGATCCAAGTGGTAGCACCAACCAAAAATCTAAAGCTAAGA
AAGGATAA

Predicted amino acid sequence of gene CP220 CPT 0052

MWFERLFVISNHIKRIKMANLLSPGIQVSEVDQSQITPVEGDSAAVFGGDFEK
GPVGVHTLISSVQELRDNYGMPNTKNYNDYYQVQNFLAYSGAIYVSRAADL
NGTPTKLDGLQFEENAYKTNVNATKVEGVKVIEADSVDVKFEKTDKFQVGQ
VLKFNDSNKEYKIKYVRNEVKQIPNPDYQPLTQLVVDPSQASAYVDEVVSYV
VTTNAESYTVETDRPDVVLVNKSNKSLTALKVGTAIVTFRATKEGSRPNTFEF
VLNVQEKEQTKLVVTPETVNILEGQTAMLNIDTDAETYSIVSKNLAIATIAED
KKTINGLRIGSCLAEVSAQANNKTETTKIINVNVITGVETDLTVSPEGPVTLHK
NEEQIFEITSSGASILETASEADKEFVTVDKTAKKVTAIKEGNAVVRVRAKAL
GADEVIKRIQIIILPEKISATVEPTEISINTDAGDQTLTVTTEATNISARVIDTSVA
NVSTQEKIITVTPLAAGNTEIEITVSSEGYSNNVITVPLTVTAVNADPSGSTNQ
KSKAKKG

Nucleotide sequence of gene CP220 CPT 0053 with the amplified region shown in blue

ATGGCAGATATCTACGATATACCAGAGTTTATAACTCAAGAAGTAACTAT
TGTTACTTTAGACAAAGAACCAGGTGAACTAAATGCAGACACTTCTGTAT
ACTTACTAGAAGGTGAATCACAACCAGATTCTAACTATATTTTAAGTCTTA
GAGGTGCTAACACTGAATTAAAACGTGGTGATATTATAGCATTTTCTGATG
TTTTAACTGATCCTAGATTCAGAATCTTAGCAATCTCTGACAGTATAGTCA
ATGGCGAAGCTTTCACAAATATTACTTATGAAGGCACAGAGGATTCTGAA
GCCATTGTTGAAGCAACTAAAGGATTTCCAGTTTATTTAGTTAAATCTACT
AAATCAGCTTGTGTTGAAGTTCCTGTAGAAGGTTCTGAAACCAAATACGA
CTCATCAGAATATGAACTTTATGATCATACCATTAGTAACTTCAATACATT
TGATGAAGATAAATTATCTAAACCATTTGTTTATAAAGATGCAAAATTAA
AAATATTTGCTAAAACACCAGGAACTTGGGGAAATAAAATTGATGTTGCA
ATAGCACACCCTGATGATTTTAACAAAGGTAAATATATCACTGATGGTAT
ACCATTAGATTCTCAATTTGATTATATTCCTTACGGTGATCAATTTGCTGTT
ATTGTTATCTACGCAAACGAAATTCAAGAATCATTTATTGTAAGTCTAGGA
TTGACTGATAAAAATGAGAAAAATGAATTTACTTATATAGAAACAATGAT
TAATGGTAAGTCAAGCTATATCTTAGTTTCTGTAAATGAAGCAGTTCAAGG
TAAACCAAAAACTTGTTTAGGTGAAGATTTACTTAAACTTGAAAATGGTA
TGGATTCAGCTCCAGGTATTGACGACATCATAGACGCTTATACAATTTTCG
ACAACAAAGAAGAAATCGATGTTGATATCTTAATTTGTAACGAAACTTAT
CCAAAAGCAGCTACTGATATTGCGATTACTCGTGGTGACTGTATAGCATTT
ATGGGTGCTCCAAAAAGTTGTTCAGTGGGCTATAAATCTACAATTGCTAAT
CAAAAAACACTTGATTTTAGAAAATCTTTAAATATAGATTCTAAATATGTA
ACTTTGTGTAGCAATTACAAATATCAATATTGTGCTGAGCTTGGTGGTTAC
AGATGGGTGAATCTAGCAGCAGATATTGCAGGTCTTAAAGCTCAAACAAA
TTATAATCAAGCTAACTGGTATGCAGCTGCTGGTCTTAACAGGGGTCTCAT
TAAAAACTGCGAGGCGTTGTCATATAGCCCAACTGGTGCGATGCGAGATA
CACTTTACAAGAATGGTATAAATCCAGTGGTTATGTTTCCAAACACTGGTG
CGGTTCTTTGGGGTCAAAAAACATTACAAACTAAAGCTTCAAGCTTCGAT
CGCGTAAACGTTGTTAGCTTGTTTAACCATTTGGAAAGATCTTTAGGTCGT
ATGTCGAAGTACAGTCTATTTGAGTTCAATGATAGTTTTACAAGAAATTAC
CTTGTAAGTATTATCAAACCTTTCTTGGCTCAAGTAAAAGCTGGTCGCGGG
ATCAGCGATTATTTAGTCATATGCGATGCATCAAACAATCCAGCAAGCGT
AATCAGTGCAAACCAACTCGTCATAGACGTATATATTAAGCCGACTTATG
TTGCAGAGTTCATTCATCTCAGATTCGTGAACGTCGGCACAAACGACTTTA
GTGTTGTTGTAAGCTAA

Predicted amino acid sequence of gene CP220 CPT 0053

MADIYDIPEFITQEVTIVTLDKEPGELNADTSVYLLEGESQPDSNYILSLRGAN
TELKRGDIIAFSDVLTDPRFRILAISDSIVNGEAFTNITYEGTEDSEAIVEATKGF
PVYLVKSTKSACVEVPVEGSETKYDSSEYELYDHTISNFNTFDEDKLSKPFVY
KDAKLKIFAKTPGTWGNKIDVAIAHPDDFNKGKYITDGIPLDSQFDYIPYGDQ
FAVIVIYANEIQESFIVSLGLTDKNEKNEFTYIETMINGKSSYILVSVNEAVQGK
PKTCLGEDLLKLENGMDSAPGIDDIIDAYTIFDNKEEIDVDILICNETYPKAAT
DIAITRGDCIAFMGAPKSCSVGYKSTIANKTLDFRKSLNIDSKYVTLCSNYKY
QYCAELGGYRWVNLAADIAGLKAQTNYNQANWYAAAGLNRGLIKNCEALS
YSPTGAMRDTLYKNGINPVVMFPNTGAVLWGQKTLQTKASSFDRVNVVSLF
NHLERSLGRMSKYSLFEFNDSFTRNYLVSIIKPFLAQVKAGRGISDYLVICDAS
NNPASVISANQLVIDVYIKPTYVAEFIHLRFVNVGTNDFSVVVS

Nucleotide sequence of gene CP220 CPT 0035c with the amplified region shown in blue

ATGTTGTCTAGAAAGATAAGAATTGATAACTTTATCTCTGATAAAGAAAA
AGAAGAAATATATCAATATTCTAAATCTCTTTCTGCTATCTACAACGAATG
TCTAGATCTTCTTAAAGAAAATTTGAATTTTAAAGATCTATCTAAAATCAC
AAAAGGTGGATCAAAAGCAACAGGCTTGCATTCAAAACATATACAGAAT
ACCTCTAGAGAGGTTATAAATGCTGTAAAATCTTATCTAGTGAAAAAGAA
GAACGATAAGTCTGCTAGATTTCCAAAATTACATAGAGAATATAGTCCTA
TCATTATGGTCGCAAATCTTTCCTCTAAGACGGAGGAGATTGTTAACTCTA
GAACTGGAGAAGCGATTCTCGAAAAGAAATACTATCCAGGTGGAGGATTT
AAACTAGAAGGTAAGAAAATAAACTTTACTTCAATAGGATTTGAATTAGA
TTTAAGTAAATGCCCTTACTATGATATAGAACTTATCAACTATGAGACTTT
AAAACAGATAGTCATCAAGATCGATGAGAATAAGAGAATAGATTGTATTT
TTGTTTTCTCAGAGAAAAAACAAGAAAAAGCACCAAATCAAAATTTTCTT
TCCATAGATCTAGGAATAAGCAGTATAGCATCTTGTTACTCAAACAAGAT
TGATTGCTTGAAGATACAAACTAAGAGATTTAAAGGTCTAGAAAGAACTA
TAAATGAGCTGAAGTCTAAAAGAGATAAGAAGAAAAAAGACTCAAGAGC
ATATAAGAAACTTAACAAAACAATCAGAAGAAAGCAAGCAAAACTAACT
AATAAAAGAAAAGACTATCTTCATAAGGCCTCAAAGACTGTAGTAGATCT
CTGTATTCTTAATGGTATAGATAACATCATTTGTGGAGATATCAAGACTAA
AAAATTAAAGAAAGACTATAAAACAAGTTTAAACAAATCAACCCAAAAT
GAAGGACTATTGAGTAGATTTAAGGGTTTCTTAAAGTATAAAGCAGAGAA
TAAAGGATTGAACTTTTTACTTGTGAATGAAGCATATACTTCTCAGACTAA
CTGCCTTACAGGAAAAAGAGAGCTAGACTCGAATCTCAGTATCAGAGAGG
TAGAATTAAGTCCAGGTTTCAAAGTTGATAGAGATATAAATTCAGCTGTC
AATATAGCCAAAATATGTGGGGATTTATGGTTATCCCATATCTTTGAGAAG
AATAGACTTCTCAAGATACAAAAAATGAATATTACTTTGTAA

Predicted amino acid sequence of gene CP220 CPT 0035c

MLSRKIRIDNFISDKEKEEIYQYSKSLSAIYNECLDLLKENLNFKDLSKITKGGS
KATGLHSKHIQNTSREVINAVKSYLVKKKNDKSARFPKLHREYSPIIMVANLS
SKTEEIVNSRTGEAILEKKYYPGGGFKLEGKKINFTSIGFELDLSKCPYYDIELI
NYETLKQIVIKIDENKRIDCIFVFSEKKQEKAPNQNFLSIDLGISSIASCYSNKID
CLKIQTKRFKGLERTINELKSKRDKKKKDSRAYKKLNKTIRRKQAKLTNKRK
DYLHKASKTVVDLCILNGIDNIICGDIKTKKLKKDYKTSLNKSTQNEGLLSRF
KGFLKYKAENKGLNFLLVNEAYTSQTNCLTGKRELDSNLSIREVELSPGFKV
DRDINSAVNIAKICGDLWLSHIFEKNRLLKIQKMNITL

Nucleotide sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0035c sequence with the differences to CP220 CPT 0035c sequence shown in red

GGTGGATCAAAAGCAACAGGCTTACATTCAAAACATATACAGAATACCTC
TAGAGAGGTTATAAACGCTGTAAAATCTTATCTAGCGAAAAAGAAGAACG
ATAAGTCTGCTAGATTTCCAAAATTACATAGAGAATATAGTCCTATCATTA
TGGTCGCAAATCTTTCCTCT

Predicted amino acid sequence obtained from CP20 genomic DNA amplified with primers designed using the CP220 CPT 0035c sequence with the differences to CP220 CPT 0035c sequence shown in red

MLSRKIRIDNFISDKEKEEIYQYSKSLSAIYNECLDLLKENLNFKDLSKITKGGS
KATGLHSKHIQNTSREVINAVKSYLAKKKNDKSARFPKLHREYSPIIMVANLS
SKTEEIVNSRTGEAILEKKYYPGGGFKLEGKKINFTSIGFELDLSKCPYYDIELI
NYETLKQIVIKIDENKRIDCIFVFSEKKQEKAPNQNFLSIDLGISSIASCYSNKID
CLKIQTKRFKGLERTINELKSKRDKKKKDSRAYKKLNKTIRRKQAKLTNKRK
DYLHKASKTVVDLCILNGIDNIICGDIKTKKLKKDYKTSLNKSTQNEGLLSRF
KGFLKYKAENKGLNFLLVNEAYTSQTNCLTGKRELDSNLSIREVELSPGFKV
DRDINSAVNIAKICGDLWLSHIFEKNRLLKIQKMNITL

Appendix 2

Nucleotide sequence of gene CPt10 CPT 0771 with the amplified region shown in blue

ATGAACTTCTACAATTTAATTTATGAAAACAAAAAATATAAACCAAAAAC
TAAAGCAGATCTTAAAGAATTAATCAATGATCTTAGTATTAAATTAAGTG
ATATAGATACTAGTGATATAACAGATTTTAATGAATTATTTTCTGGAACAA
AAAGAACTGATTTCAAAGGTATTGGCACTTGGAATACCAGCAATGTTGTT
ACTGCTAGACGTTGTTTTTATAATTTAAAAGATTTTAATGAAGATATTAGT
AAATGGAATACTGCTAAACTTCAAGATGCTAGAGAAATGTTCTTCAAA
TGCAAATCATTTAATCAAAATTTAAATTCTTGGAATGTTGGAAATGTTAAG
AATATGAATAAAATGTTTTATGAATGTACTAATTTTAAACAAATATTAAAC
AAATGGAAAGTTGATAAATGTGAGAACTTTTCAGCAATGTTTTTAAATGTT
AAATACGTAGAGGAACACTTTAAAGAATTTAAATGGAATACTAGAAATGC
TGACGGTTATTCAGCAAACCCATTCAAAGCTTAG

Predicted amino acid sequence of gene CPt10 0771

MNFYNLIYENKKYKPKTKADLKELINDLSIKLSDIDTSDITDFNELFSGTKRTD
FKGIGTWNTSNVVTARRCFYNLKDFNEDISKWNTAKLQDAREMFFKCKSFN
QNLNSWNVGNVKNMNKMFYECTNFKQILNKWKVDKCENFSAMFLNVKYV
EEHFKEFKWNTRNADGYSANPFKA

Nucleotide sequence of gene CPt10 1761 showing the amplified region in blue

ATGTTATCTAGAAAGATAAGAATTGATAGCTTTATCTCTGATAAAGAAAA
AGAAGAAATTTATCAGTATTCTAAATCTCTTTCTACCGTCTATAATGAATG
TCTAGATCTTCTTAAAGAAAATTTGAATTTTAAAGATCTATCTAAAATCAC
AAAAGGTAGATCAAAAGCAACAGGCTTACATTCAAAACATATACAGAAT
ACCTCTAGAGAGGTTATAAACGCTGTAAAATCTTATCTAGCGAAAAAGAA
GAACGATAAGTCTGCTAGATTTCCAAAATTACATAGAGAATATAGTCCT
ATCATTATGGACATAAATCTTTCCTCTAAGACGGAGGAGATTGTTAACTCT
AGAACTGGAGAAGTGATTCTCGAAAAGAAATACTATCCAGGTGGAGGATT
TAAACTAGAAGGTAAGAAAATAAACTTTACTTCAATAGGATTTGAATTAG
ATTTAAGTAAATGCCCTTACTATGATATAGAACTTATCAACTATGAGACTT
TAAAACAGATAGTCGTCAAGATTGATGAGAATAAGAGAATAGATTGTATT
TTTGTTTTCTCAGAGAAAATACAAGAAAAAGCACTAAATCAAAATTTTCTT
TCTATAGATCTAGGAGTAAGCAGTATAGCATCTTGCTACTCAAACAAGAT
TGATTGCTTGAAGATACAAACTAAGAGATTTAAAGGTCTAGAAAGAACTA
TAAATGAGCTGAAGTCTAAAAGAGATAAGAAGAAAAAAGACTCAAGAGC
ATATAAGAAACTTAACAAAACAATCAGAAGAAAGCAAGCAAAACTAACT
AATAAAAGAAAAGACTATCTCCATAAGACCTCAAAAACTATAGTAGATCT
CTGCATTCTTAATGGTATAGATAACATCATTTGTGGAGATATCAAGACT
AAAAAATTAAAGAAAGACTATAAAACAAGTTTAAACAAATCAACTCAAA
ATGAAGGACTATTGAGTAGATTTAAGGGTTTCTTAAAGTATAAAGCAGAG
AATAAAGGGTTGAACTTTTTACTTGTGAATGAAGCATATACTTCTCAGACT
AACTGTCTTACAGGAAAAAGAGAGCTAGACTCGAATCTTGGTATTAGAGA
GGTAGAATTAAGTCCAGGTTTCAAAGTTGATAGAGATATAAATTCAGCTG
TCAATATAGCCAAAATATGTGGGGATTTATGGTTATCCCATATCTTTGAG
AAGAATAGACTTCTCAAGATACAAAAAATGAATATTACTTTGTAA

Predicted amino acid sequence of gene CPt10 1761

MLSRKIRIDSFISDKEKEEIYQYSKSLSTVYNECLDLLKENLNFKDLSKITKGRS
KATGLHSKHIQNTSREVINAVKSYLAKKKNDKSARFPKLHREYSPIIMDINLSS
KTEEIVNSRTGEVILEKKYYPGGGFKLEGKKINFTSIGFELDLSKCPYYDIELIN
YETLKQIVVKIDENKRIDCIFVFSEKIQEKALNQNFLSIDLGVSSIASCYSNKID
CLKIQTKRFKGLERTINELKSKRDKKKKDSRAYKKLNKTIRRKQAKLTNKRK
DYLHKTSKTIVDLCILNGIDNIICGDIKTKKLKKDYKTSLNKSTQNEGLLSRFK
GFLKYKAENKGLNFLLVNEAYTSQTNCLTGKRELDSNLGIREVELSPGFKVD
RDINSAVNIAKICGDLWLSHIFEKNRLLKIQKMNITL

Nucleotide sequence obtained from CP220 genomic DNA amplified with primers designed using the CPt10 1761 sequence with the differences to CPt10 1761 sequence shown in red

GATCAAAAGCAACAGGCTTACATTCAAAACATATACAGAATACCTCTAGA
GAGGTTATAAATGCTGTAAAATCTTATCTAGTGAAAAAGAAGAACGATAA
GTCTGCTAGATTTCCAAAATTACATAGAGAATATAGTCCTATCATTATGGT
CGCAAATCTTTCCTCTAAGACGGAGGAGA

Predicted amino acid sequence of CP220 amplified with CPt10 1761 primers showing the differences to CPt10 1761 in red

MLSRKIRIDSFISDKEKEEIYQYSKSLSTVYNECLDLLKENLNFKDLSKITKGRS
KATGLHSKHIQNTSREVINAVKSYLAKKKNDKSARFPKLHREYSPIIMDINLSS
KTEEIVNSRTGEVILEKKYYPGGGFKLEGKKINFTSIGFELDLSKCPYYDIELIN
YETLKQIVVKIDENKRIDCIFVFSEKIQEKALNQNFLSIDLGVSSIASCYSNKID
CLKIQTKRFKGLERTINELKSKRDKKKKDSRAYKKLNKTIRRKQAKLTNKRK
DYLHKTSKTIVDLCILNGIDNIICGDIKTKKLKKDYKTSLNKSTQNEGLLSRFK
GFLKYKAENKGLNFLLVNEAYTSQTNCLTGKRELDSNLGIREVELSPGFKVD
RDINSAVNIAKICGDLWLSHIFEKNRLLKIQKMNITL

Nucleotide sequence of gene CPt10 0591 showing the amplified region in blue

TTATTATACACAATTGATGAAAATAATTTCATCCAAAAATTATTATATATA
AACGATAATAATATAAACTTTAAAGGTTATCATAATACTATTGCAAACCA
GAAATTATTAAAAAAAATTAAGACAGCTAGTTACTAACAATTTAAAAATAG
AATTAAAACATTTTATAAGACTCTTAGCAAGTGGAAAAATTTTCTGTCCAG
ATTGTGGTGATTTATTAGAAGCTGATCTTAATTGTAATTGTAAAAAGATAA
AATGTAGTTGTTGTGACTACAAGGCCAATTCAATAAAAGGTATTAAA
AATCATTATACAACAAAACATAAAACAAATTACGTTTACAAACCTACAGT
GTATTGTTGTTATTGTGGTGAAAAATTAGCAGTTAATGAATACGGGTATGC
TGGACAATGTTTTAATAAAGACTGCGATTCATTTAAATTTAGAATAGAAA
CTTTTAGAACAAATATCACAAAAACTGTTAACAATTATACTAATATAAAA
AGGATAGTAAGACACGTTCGGTATAGCCGAGCTGCTAAACTGAGAGAAGT
TATGATGTGCAATACATACATAGGAGATAAAACGAAAAAAGAATTAGCC
GGTATTAAAGCGGGTGCTAAATTATCAGTTATAATGAAAGAAAAAATAAA
AAATGGTGAATTTACACCTTGTGTTACTAATAGCTGGTGCAGAAGCAGAA
TATTATACAAGGGCAACGCTTTTCGCAGTTCATTCGAAGTATTGTTCAAAT
TATTGGATACAGAAGACAAACTTTTATATGAAAAAACTGTAATACCTTAC
GACCACTACGGAGTAGCCCGTAATTATATAGTTGATTTTACCGATTTTGAT
AACAAAATATTATATGAAATTAAACCAAAATCTGAGATAAACAACGAT
TTAAACAAAATAAAAGAAAATGCTGCAATTAAGTGGTGTAAAAACAATG
GATTTGTATATAAAATAATAACCGAAGATTATTTAAAAAAATATAAAAAT
AAAATTGTAGACAACTTCTTGGCTAATAAGGTGGCTTTTGACAACGAAAG
TCTACGTAAAATTAATAATTCGTTAAAAGGATTAAAATGA

Predicted amino acid sequence of gene CPt10 0591

LLYTIDENNFIQKLLYINDNNINFKGYHNTIANQKLLKKLRQLVTNNLKIELKH
FIRLLASGKIFCPDCGDLLEADLNCNCKKIKCSCCDYKANSIKGIKNHYTTKH
KTNYVYKPTVYCCYCGEKLAVNEYGYAGQCFNKDCDSFKFRIETFRTNITKT
VNNYTNIKRIVRHVRYSRAAKLREVMMCNTYIGDKTKKELAGIKAGAKLSVI
MKEKIKNGEFTPCVTNSWCRSRILYKGNAFRSSFEVLFKLLDTEDKLLYEKTV
IPYDHYGVARNYIVDFTDFDNKILYEIKPKSEINNDLNKIKENAAIKWCKNNG
FVYKIITEDYLKKYKNKIVDNFLANKVAFDNESLRKINNSLKGLK

Nucleotide sequence of gene CPt10 1471 showing the amplified region in blue

ATGTCAGTTGAAATAATTAATGATGATTGTTTAAACATATTGGAAAATATA
AGAAATGTTGATTTAATTATAACAGACCCACCATACTTCGTAATTCCTAAA
GGTAAAAAAAACAAATAATGGATATGATAATTTTAAATGGGATAGTTTTGA
CAATATGGATCATTTTTTAAAATTTACAAAAGAATGGTTTGATTTATGTTA
TAAAAAATTAAATAATGATTCATTTATGTATATATTTTGGAGTCAAAAGTA
TTTTTCATATGGTTTTGAAATTTTTAATCCAAATCGTGTTTTGTTATGGCAC
TATAGAAATTTAGTACTCGGTGGTAATGGAGATTTTGCATATGATTACGAA
CCTATTTTTGTTATAAAGAAAGGTAATCCTAAATTAATAAAAGGCAAACA
TAGTTCGATTTTAAATTTTACAAAACCTCAGAGTAATTTTAAGGCCGACAA
ACTCGTTCACCCAACACAAAAACCATTAAAATTAATAGAATACTTGATAT
CAATATCAAATCTTAAAGAAAATGCTGTGATTTTGGATCCGTTTGGTGGTG
CAGGGACAACAGCGTTAGCATCAAATAATTTAAAATATGATTGCATTACT
ATAGAAAAAGAGACTGGGTATTGTAACTTAATAAACAATAGATTGTTAAT
AAAGTAA

Predicted amino acid sequence of gene CPt10 1471

MSVEIINDDCLNILENIRNVDLIITDPPYFVIPKGKKTNNGYDNFKWDSFDNM
DHFLKFTKEWFDLCYKKLNNDSFMYIFWSQKYFSYGFEIFNPNRVLLWHYRN
LVLGGNGDFAYDYEPIFVIKKGNPKLIKGKHSSILNFTKPQSNFKADKLVHPT
QKPLKLIEYLISISNLKENAVILDPFGGAGTTALASNNLKYDCITIEKETGYCNL
INNRLLIK

Appendix 3

Annotation of CPX Bacteriophage

Source Feature	Location	Direction	Note
CDS	<1..1393	Reverse (R)	Similar to AEA86399.1 Hef53 [Campylobacter phage NCTC12673]
CDS	1454..2191	R	Putative gp2 DNA end protector protein; similar to AEA86400.1 gp2 DNA end protector protein [Campylobacter phage NCTC12673]
CDS	2200..2553	R	Hypothetical protein
CDS	2602..3318	R	Similar to YP_004322538.1 terminase DNA packaging enzyme large subunit [Prochlorococcus phage P-HM1]
CDS	3355..3828	R	Similar to AEA86401.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	3831..4487	R	Similar to AEA86402.1 Thymidylate synthetase [Campylobacter phage NCTC12673]
CDS	4683..5990	Forward (F)	Similar to AEA86404.1 Hef59 [Campylobacter phage NCTC12673]
CDS	6149..7972	F	Similar to AEA86405.1 Hef60 [Campylobacter phage NCTC12673]
CDS	7987..9897	R	Similar to AEA86406.1 gp18 tail sheath protein [Campylobacter phage NCTC12673]
CDS	10061..10408	R	Similar to AEA86407.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	10398..11351	R	Similar to AEA86408.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	11373..12695	R	Similar to AEA86409.1 gp30 DNA ligase [Campylobacter phage NCTC12673]
CDS	12867..13166	R	Similar to AEA86411.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	13156..13656	R	Similar to AEA86412.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	13674..14066	R	Similar to AEA86413.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	14166..14924	R	Similar to AEA86414.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	14935..15567	R	Similar to AEA86415.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	15612..16694	F	Similar to AEA86416.1 Hef71 [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	16714..17637	R	Similar to YP_003785013.1 ribonucleotide-diphosphate reductase subunit alpha [Brachyspira pilosicoli 95/1000]; pfam02867, ribonucleotide reductase barrel domain: aa 11..290, score 5.64e-62
CDS	17663..18982	R	Similar to AEA86418.1 Hef74 [Campylobacter phage NCTC12673]
CDS	19079..20476	R	Similar to AEA86419.1 gp23 major capsid protein [Campylobacter phage NCTC12673]
CDS	20507..21253	R	Similar to AEA86420.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	21264..21893	R	Similar to AEA86421.1 gp21 prohead core scaffold and protease [Campylobacter phage NCTC12673]
CDS	22058..23764	R	Similar to AEA86423.1 gp20 portal vertex protein [Campylobacter phage NCTC12673]
CDS	23809..24510	F	Similar to AEA86424.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	24513..25244	F	Similar to AEA86425.1 gp13 neck protein [Campylobacter phage NCTC12673]
CDS	25271..26599	F	Similar to CBJ94203.1 Putative phage DNA packaging protein (terminase) [Campylobacter phage CPt10] & CBJ93810.1 putative phage DNA packaging protein (terminase) [Campylobacter phage CP220]
CDS	26628..26849	F	Similar to AEA86426.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	26852..27583	F	Similar to AEA86427.1 gp19 tail tube protein III [Campylobacter phage NCTC12673]
CDS	27578..29023	R	Similar to AEA86428.1 Hef85 [Campylobacter phage NCTC12673]
CDS	28995..29762	R	Similar to AEA86429.1 phosphatidylserine decarboxylase [Campylobacter phage NCTC12673]
CDS	29773..30069	R	Similar to AEA86430.1 co-chaperonin GroES [Campylobacter phage NCTC12673]
CDS	30125..30634	R	Similar to AEA86431.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	30637..31134	R	Similar to AEA86432.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	31345..33708	R	Similar to AEA86434.1 putative DEAD/DEAH box helicase [Campylobacter phage NCTC12673]
CDS	33765..35156	R	Similar to AEA86435.1 DNA helicase UvsW [Campylobacter phage NCTC12673]
CDS	35325..35744	R	Similar to AEA86436.1 putative repair and recombination protein [Campylobacter phage NCTC12673]
CDS	35746..36471	R	Similar to AEA86437.1 hypothetical protein [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
Misc feature	36262..36471	R	Signal peptide predicted for CPX_0380 by signal P 3.0 (signal peptide probability: 0.982) with cleavage site probability 0.920 between aa positions 17 and 18
CDS	36643..38025	R	Similar to AEA86438.1 hypothetical protein [Campylobacter phage NCTC12673]
tRNA-Tyr	38268..38350	R	codon recognized: UAC; cove score 58.55 predicted by tRNAscan-SE 1.21" /anticodon=(pos:complement(38314..38316),aa:Tyr
tRNA-Arg	38364..38437	R	Codon recognized: AGA; cove score 66.75 predicted by tRNAscan-SE 1.21" anticodon=(pos:complement(38404..38406),aa:Arg)
tRNA-Asn	38450..38522	R	Codon recognized: AAC; cove score 67.75 predicted by tRNAscan-SE 1.21" /anticodon=(pos:complement(38488..38490),aa:Asn
tRNA-Leu	38875..38949	R	Codon recognized: UUA; cove score 58.26 predictedby tRNAscan-SE 1.21" /anticodon=(pos:complement(38912..38914),aa:Leu
tRNA-Met	38958..39029	R	Codon recognized: AUG; cove score 79.75 predicted by tRNAscan-SE 1.21" /anticodon=(pos:complement(38995..38997),aa:Met
CDS	39213..39860	R	Similar to AEA86439.1 hypothetical protein [Campylobacter phage NCTC12673
CDS	39841..41019	R	Similar to AEA86440.1 putative methylase [Campylobacter phage NCTC12673
CDS	41766..42110	R	Similar to AEA86442.1 hypothetical protein [Campylobacter phage NCTC12673]"
CDS	42167..42781	R	Similar to AEA86443.1 hypothetical protein [Campylobacter phage NCTC12673
CDS	43284..45947	R	Similar to AEA86445.1 gp43 DNA polymerase [Campylobacter phage NCTC12673
CDS	45992..46921	R	Similar to AEA86446.1 putative methyltransferase [Campylobacter phage NCTC12673
CDS	47017..47430	R	Similar to AEA86447.1 hypothetical protein [Campylobacter phage NCTC12673
CDS	47427..47867	R	Similar to AEA86448.1 hypothetical protein [Campylobacter phage NCTC12673]; proteim motif: pfam07087, protein of unknown function (DUF1353); This family consists of several hypothetical bacterial proteins of around 100 residues: aa 24..125, score 2.17e-21"
CDS	47869..48372	R	Similar to AEA86449.1 Thymidine kinase [Campylobacter phage NCTC12673]; proteim motif: pfam00265, thymidine kinase: aa 2..161, score 2.20e-17

Source Feature	Location	Direction	Note
CDS	48558..49502	R	Similar to AEA86451.1 gp61 DNA primase subunit [Campylobacter phage NCTC12673]; proteim motif: PHA02540, DNA primase; Provisional: aa 24..314, score 1.34e-22
CDS	49486..50010	R	Similar to AEA86452.1 hypothetical protein [Campylobacter phage NCTC12673
Misc feature	49801..50010	R	Signal peptide predicted for CPX_0500 by signal P 3.0 (signal peptide probability: 0.980) with cleavage site probability 0.473 between aa positions 26 and 27
CDS	50082..50405	R	Hypothetical protein
CDS	50491..50946	F	Similar to AEA86453.1 gp3 tail completion and sheath stabilizer protein [Campylobacter phage NCTC12673]
CDS	50947..51228	R	Similar to AEA86454.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	51414..52079	R	Similar to AEA86456.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	52116..52820	R	Similar to AEA86457.1 hypothetical protein Campylobacter phage NCTC12673]
CDS	52947..53582	R	Similar to AEA86457.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	53778..54377	R	Similar to AEA86460.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	54523..54732	R	Hypothetical protein
CDS	54695..56032	R	Similar to AEA86461.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	56056..57660	R	Similar to AEA86462.1 gp46 recombination endonuclease [Campylobacter phage NCTC12673]; proteim motif: PHA02562, endonuclease subunit; Provisional: aa 1..532, score 4.19e-44
CDS	57744..58064	R	Similar to AEA86463.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	58123..59118	R	Similar to AEA86464.1 gp47 recombination endonuclease [Campylobacter phage NCTC12673]; protein motif: pfam00149, this family includes a diverse range of phosphoesterases, including protein phosphoserine phosphatases, nucleotidases, sphingomyelin phosphodiesterases and 2'-3' cAMP phosphodiesterases as well as nucleases such as bacterial SbcD or yeast MRE11: aa 5..180, score 5.48e-05
CDS	59106..59975	R	Similar to AEA86465.1 gp55 Sigma factor for T4 late transcription [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	60067..61029	F	Similar to AEA86466.1 gp23 major head protein II [Campylobacter phage NCTC12673]; proteim motif: pfam07068, major capsid protein Gp23; This family contains a number of major capsid Gp23 proteins approximately 500 residues: aa 68..147, score 2.69e-03
CDS	61039..61686	F	Similar to AEA86467.1 gp14 head completion protein [Campylobacter phage NCTC12673]
CDS	61683..63449	R	Similar to AEA86399.1 Hef53 [Campylobacter phage NCTC12673]
CDS	63558..64589	F	Similar to AEA86469.1 gp15 tail sheath stabilizer and completion protein [Campylobacter phage NCTC12673]
CDS	64586..65041	F	Similar to AEA86470.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	65038..65589	R	Hypothetical protein
CDS	65800..66102	R	Similar to AEA86472.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	66388..66771	R	Similar to AEA86474.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	66894..67334	R	Similar to AEA86475.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	67569..68228	R	Similar to AEA86476.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	68800..69033	R	Similar to AEA86480.1 hypothetical protein [Campylobacter phage NCTC12673]
Misc feature	68824..69033	R	Signal anchor predicted for CPX_0740 by signal P 3.0 (signal anchor probability: 0.485) with cleavage site probability 0.217 between aa positions 20 and 21
CDS	69173..69700	R	Similar to CBJ94264.1 hypothetical protein [Campylobacter phage CPt10] & AEA86481.1 putative exonuclease [Campylobacter phage NCTC12673]
CDS	69895..70311	R	Similar to AEA86482.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	70308..70649	R	Similar to AEA86483.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	70633..71151	R	Similar to AEA86484.1 hypothetical protein [Campylobacter phage NCTC12673] & CBJ94266.1 hypothetical protein [Campylobacter phage CPt10]
CDS	71148..71513	R	Similar to AEA86485.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	71570..72295	R	Similar to AEA86486.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	72306..72590	R	Similar to AEA86487.1 hypothetical protein [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	72624..73478	R	Similar to AEA86488.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	73517..74059	R	Similar to AEA86489.1 hypothetical protein [Campylobacter phage NCTC12673] & EFV09831.1 hypothetical protein CSS_0089 [Campylobacter jejuni subsp. jejuni 305]
CDS	74217..74513	R	Similar to CBJ94273.1 hypothetical protein [Campylobacter phage CPt10]
CDS	74428..74649	R	Similar to CBJ94272.1 hypothetical protein [Campylobacter phage CPt10]
CDS	74651..75265	R	Similar to CBJ94271.1 hypothetical protein [Campylobacter phage CPt10] & AEA86490.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	75401..75649	R	Similar to AEA86491.1 hypothetical protein [Campylobacter phage NCTC12673] & CBJ94270.1 hypothetical protein [Campylobacter phage CPt10]
CDS	76220..76627	R	Similar to AEA86494.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	77454..77897	R	Similar to AEA86495.1 gp4 head completion protein [Campylobacter phage NCTC12673]
Repeat region	77902..77941	F	predicted by Tandem Repeats Finder
CDS	77917..78627	R	Similar to AEA86496.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	78782..79078	R	Similar to AEA86497.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	79081..80202	R	Similar to AEA86498.1 rnlA; RNA ligase [Campylobacter phage NCTC12673]
CDS	80422..80715	R	Similar to AEA86500.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	80722..80961	R	Similar to AEA86501.1 hypothetical protein [Campylobacter phage NCTC12673]
Misc feature	80752..80961	R	Signal peptide predicted for CPX_0930 by signal P 3.0 (signal peptide probability: 0.999) with cleavage site probability 0.931 between aa positions 16 and 17
CDS	81532..82593	R	Similar to AEA86504.1 putative poly A polymerase [Campylobacter phage NCTC12673]
CDS	82633..84018	R	Similar to AEA86505.1 ribonucleotide reductase large subunit [Campylobacter phage NCTC12673]
CDS	83993..85126	R	Similar to AEA86506.1 ribonucleotide reductase small subunit [Campylobacter phage NCTC12673]
CDS	85139..85498	R	Similar to AEA86507.1 hypothetical protein [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
Misc feature	85289..85498	R	Signal anchor predicted for CPX_0970 by signal P 3.0 (signal anchor probability: 0.7725) with cleavage site probability 0.151 between aa positions 28 and 29
CDS	85491..85862	R	Similar to AEA86508.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	87603..88646	R	Similar to AEA86511.1 RecA [Campylobacter phage NCTC12673]
CDS	88648..88884	R	Similar to AEA86512.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	88941..89240	R	Similar to AEA86513.1 hypothetical protein [Campylobacter phage NCTC12673]
Misc feature	89031..89240	R	Signal peptide predicted for CPX_1010 by signal P 3.0 (signal peptide probability: 0.997) with cleavage site probability 0.695 between aa positions 23 and 24
CDS	89203..89547	R	Similar to AEA86514.1 hypothetical protein [Campylobacter phage NCTC12673]
Misc feature	89338..89547	R	Signal anchor predicted for CPX_1020 by signal P 3.0 (signal anchor probability: 0.700) with cleavage site probability 0.031 between aa positions 27 and 28
CDS	89551..90219	R	Similar to AEA86349.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	90232..91116	R	Similar to AEA86351.1 gp44 sliding clamp loader subunit [Campylobacter phage NCTC12673]
CDS	91241..91798	R	Similar to AEA86352.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	92061..92507	R	Similar to AEA86354.1 putative dUTP pyrophosphatase [Campylobacter phage NCTC12673]
CDS	92495..92887	R	Similar to AEA86355.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	92899..94167	R	Similar to AEA86356.1 putative NAD(FAD)-utilizing dehydrogenase [Campylobacter phage NCTC12673]
CDS	94220..94624	R	Similar to AEA86357.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	94858..95424	R	Similar to AEA86359.1 hypothetical protein [Campylobacter phage NCTC12673]
Misc feature	95215..95424	R	Signal peptide predicted for CPX_1100 by signal P 3.0 (signal peptide probability: 0.982) with cleavage site probability 0.369 between aa positions 21 and 22
CDS	95443..96078	R	Similar to AEA86360.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	96292..97608	R	Putative DNA topoisomerase II
CDS	97643..99268	R	Similar to AEA86361.1 Hef15 [Campylobacter phage NCTC12673]:
CDS	99278..101128	R	Similar to AEA86362.1 topoisomerase II large subunit [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	101205..102407	R	Similar to AEA86363.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	102458..102718	R	Similar to AEA86364.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	102773..103174	F	Similar to AEA86365.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	103192..103710	R	Similar to AEA86366.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	103743..104459	R	Similar to AEA86367.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	104430..105083	F	Similar to AEA86368.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	105269..105742	F	Similar to AEA86370.1 hypothetical protein [Campylobacter phage NCTC12673]"
CDS	105735..108350	F	Similar to AEA86371.1 putative peptidase [Campylobacter phage NCTC12673]"
Repeat region	107864..107974	F	Predicted by Tandem Repeats Finder
CDS	108352..109308	F	Similar to AEA86372.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	109324..109677	F	Similar to AEA86373.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	109685..110446	F	Similar to AEA86374.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	110467..110928	F	Similar to AEA86375.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	110918..112054	F	Similar to AEA86376.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	112051..112737	F	Similar to AEA86377.1 gp5 baseplate hub subunit and tail lysozyme [Campylobacter phage NCTC12673]
CDS	112724..113065	R	Similar to AEA86378.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	113049..113336	R	Similar to AEA86379.1 putative gp5.4 conserved hypothetical protein [Campylobacter phage NCTC12673]
CDS	113320..114942	R	similar to AEA86380.1 Hef34 [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	114954..115400	R	Similar to AEA86381.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	115460..116032	R	Similar to AEA86382.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	116049..116591	R	Similar to AEA86383.1 gp19 tail tube protein [Campylobacter phage NCTC12673]
CDS	116604..117182	R	Similar to AEA86384.1 gp19 tail tube protein II [Campylobacter phage NCTC12673]
CDS	117184..117744	R	Similar to AEA86385.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	117728..119284	R	Similar to AEA86386.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	119281..121374	R	Similar to AEA86388.1 gp6 baseplate wedge subunit [Campylobacter phage NCTC12673]
CDS	121753..122463	R	Similar to AEA86390.1 gp45 sliding clamp protein [Campylobacter phage NCTC12673]
CDS	122512..123495	R	Similar to AEA86391.1 Aminidotransferase [Campylobacter phage NCTC12673]
CDS	123576..124817	R	Similar to AEA86392.1 gp41 DNA primase-helicase subunit [Campylobacter phage NCTC12673]
CDS	124817..125539	R	Similar to AEA86393.1 hypothetical protein [Campylobacter phage NCTC12673]
CDS	125675..127576	R	Similar to AEA86394.1 hypothetical protein; putative tail fibre [Campylobacter phage NCTC12673]
CDS	127570..128502	R	Similar to AEA86394.1 hypothetical protein; putative tail fibre [Campylobacter phage NCTC12673]

Source Feature	Location	Direction	Note
CDS	128483..129772	R	Similar to AEA86394.1 hypothetical protein; putative tail fibre [Campylobacter phage NCTC12673]"
CDS	129823..130848	R	Similar to AEA86395.1 putative RNaseH [Campylobacter phage NCTC12673]
CDS	130966..131352	R	Similar to AEA86397.1 gp62 clamp loader subunit [Campylobacter phage NCTC12673]
CDS	131404..132321	R	Similar to AEA86398.1 gp32 ssDNA binding protein [Campylobacter phage NCTC12673]

Appendix 4

Nucleotide Sequence of gene CP220 CPT 0075 cloned in to TOPO vector with *Eco*RI restriction sites highlighted in yellow, the introduced *Nde*I restriction site is highlighted in green and the *Bam*HI site is highlighted in blue

GAATTCGCCCTTTTCATATGCCTCCTTTAACTAGAGTTGGTGTTGATT
TTAGTACAGGACATTCTTCATTCCCACCTAATGTAGTTTCGAGTGGT
TCTACGAATGTCTTGACTAATTCAATTAGTACAGTTAGACAAGGTGA
TCCTATAATACCACACGGAAGTCCTAGCCCGTCACCACCACACGGT
GGGAGTATTGCTACAGGTTCTGGGACTGTTATGGTTAATTCAAAACC
TGCTTGTAGAATAGGTGATGCCATTAGTTGTGGCCAAGCTGTAGCA
CAAGGATCTGGAAATGTTATTTGTGGATAAGGATCCAAAAGGGCGA
ATTC

Nucleotide Sequence of gene CP220 CPT 120 cloned in to TOPO vector with *Eco*RI restriction sites highlighted in yellow, the introduced *Nde*I restriction site is highlighted in green and the *Bam*HI site is highlighted in blue

GAATTCGCCCTTAACATATGAATTACGATAAACTGAATAAAATGGG
AATAATTTTGATTATTATTTTATCAGTTGTTTATTTTATGCTTGATAT
TAATAATACAAAAGTTAAAAATTTAGAATTTAAAATTCAAGATCTT
CAAATAGAACTTAATAAAACCAAAAAAGAATTAAATGATACCAAA
ATTAATTTAAATCATTTAAGTTCTAAAGTTCAAGATTTAAAAATATC
TTTAATGAAAGATATGTCGTCAATGTATCACTTAAGTGATAAACAA
CAATCTCTAATACTTGCTGAAATATGGAAACAATCTAAAAAATACA
AAATAAACCCAGCATTTTTATACGCAGTATTATGGAAAGAATCAAG
ATTTAGAAACGACGTTATTCATAAACCTACTTATGTTAGAACACTTA
AAAAAGAGATACAAGCTCAAGGTATGGGTGCTATTGTTTGGGATTT
TTGGGGAGATAAACTTAAGTCTAATACAAGTTTAAAATCTAAAAAA
GATCTTAAAAATTGGAAAAAGAATATAGAAGGGACTGCATATATAC
TTAGTTATTTGAAATCTTTACCAAAGATATCTAATACAAAAAATAAG
TACGAATCAGCAGCTTCAAGATACTACGGAAAATATCAAGCAAATT
ACGTGAATAAAACAATGTCAAAATTTAATAAACTAAACTCTTAATA
CGGATCCTTAAGGGCGAATTC

Appendix 5

Nucleotide Sequence of gene CP220 CPT 0075 cloned in to pET 3a vector with ribosomal binding site highlighted in pink, the introduced *Nde*I restriction site is highlighted in green and the *Bam*HI site is highlighted in blue

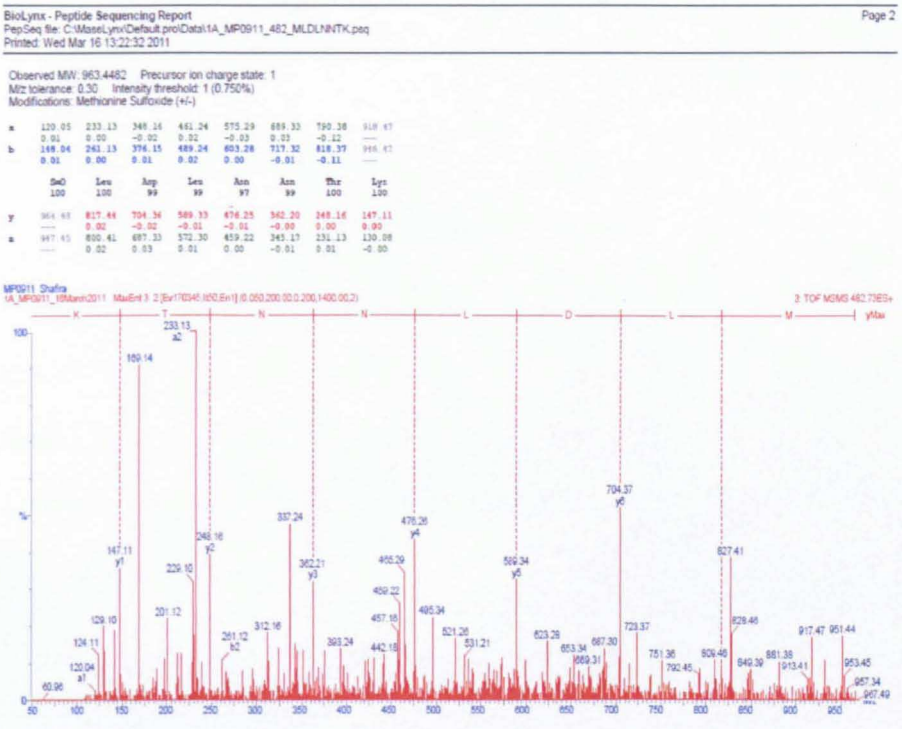
ATTTTGTTTAACTTTAAGAAGGAGATATACATATGCCTCCTTTAACT
AGAGTTGGTGTTGATTTTAGTACAGGACATTCTTCATTCCCACCTAA
TGTAGTTTCGAGTGGTTCTACGAATGTCTTGACTAATTCAATTAGTA
CAGTTAGACAAGGTGATCCTATAATACCACACGGAAGTCCTAGCCC
GTCACCACCACACGGTGGGAGTATTGCTACAGGTTCTGGGACTGTT
ATGGTTAATTCAAAACCTGCTTGTAGAATAGGTGATGCCATTAGTTG
TGGCCAAGCTGTAGCACAAGGATCTGGAAATGTTATTTGTGGATAA
GGATCCGGCTGCT

Nucleotide Sequence of gene CP220 CPT 120 cloned in to pET 3a vector with ribosomal binding site highlighted in pink, the introduced *Nde*I restriction site is highlighted in green and the *Bam*HI site is highlighted in blue

CTTTAAGAAGGAGATATACATATGAATTACGATAAACTGAATAAAA
TGGGAATAATTTTGATTATTATTTTATCAGTTGTTTATTTTATGCTTG
ATATTAATAATACAAAAGTTAAAAATTTAGAATTTAAAATTCAAGA
TCTTCAAATAGAACTTAATAAAACCAAAAAAGAATTAAATGATACC
AAAATTAATTTAAATCATTTAAGTTCTAAAGTTCAAGATTTAAAAAT
ATCTTTAATGAAAGATATGTCGTCAATGTATCACTTAAGTGATAAAC
AACAATCTCTAATACTTGCTGAAATATGGAAACAATCTAAAAAATA
CAAAATAAACCCAGCATTTTTATACGCAGTATTATGGAAAGAATCA
AGATTTAGAAACGACGTTATTCATAAACCTACTTATGTTAGAACACT
TAAAAAAGAGATACAAGCTCAAGGTATGGGTGCTATTGTTTGGGAT
TTTTGGGGAGATAAACTTAAGTCTAATACAAGTTTAAAATCTAAAA
AAGATCTTAAAAATTGGAAAAAGAATATAGAAGGGACTGCATATAT
ACTTAGTTATTTGAAATCTTTACCAAAGATATCTAATACAAAAAATA
AGTACGAATCAGCAGCTTCAAGATACTACGGAAAATATCAAGCAAA
TTACGTGAATAAAACAATGTCAAAATTTAATAAACTAAACTCTTAA
TACGGATCCGGCTG

Appendix 6

MS peptide sequencing of CP220 CPT 120 expressed protein band in Rosetta 2 (DE3) pLys S



Appendix 7

Nucleotide Sequence of His tagged gene CP220 CPT 120 cloned in to TOPO vector with *Eco*RI restriction sites highlighted in yellow, the introduced *Nde*I restriction site is highlighted in green and the *Bam*HI site is highlighted in blue and the His tag is highlighted in pink

GAATTCGCCCTTAACATATGCACCACCACCACCACCACATGAATTA
CGATAAACTGAATAAAATGGGAATAATTTTGATTATTATTTTATCAG
TTGTTTATTTTATGCTTGATATTAATAATACAAAAGTTAAAAATTTA
GAATTTAAAATTCAAGATCTTCAAATAGAACTTAATAAAACCAAAA
AAGAATTAAATGATACCAAAATTAATTTAAATCATTTAAGTTCTAA
AGTTCAAGATTTAAAAATATCTTTAATGAAAGATATGTCGTCAATGT
ATCACTTAAGTGATAAACAACAATCTCTAATACTTGCTGAAATATG
GAAACAATCTAAAAAATACAAAATAAACCCAGCATTTTTATACGCA
GTATTATGGAAAGAATCAAGATTTAGAAACGACGTTATTCATAAAC
CTACTTATGTTAGAACACTTAAAAAAGAGATACAAGCTCAAGGTAT
GGGTGCTATTGTTTGGGATTTTTGGGGAGATAAACTTAAGTCTAATA
CAAGTTTAAAATCTAAAAAAGATCTTAAAAATTGGAAAAAGAATAT
AGAAGGGACTGCATATATACTTAGTTATTTGAAATCTTTACCGAAG
ATATCTAATACAAAAAATAAGTACGAATCAGCAGCTTCAAGATACT
ACGGAAAATATCAAGCAAATTACGTGAATAAAACAATGTCAAAATT
TAATAAACTAAACTCTTAATACGGATCCTTAAGGGCGAATTC